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13740

On the Use of Sulfanilamide in Measurement of Body Water
in the Dog.*

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Sulfanilamide has been advanced for use in the measurement of the total water content of dogs.¹ The suggestion was based upon the

* This investigation has been aided by a grant from the John and Mary R. Markle Foundation.

demonstration that its volume of distribution was equal to that of urea as well as to the total water content of animals, as measured by desiccation. Unfortunately, the whole blood concentration was used in calculating the volume of distribution of sulfanilamide, thus introducing an error due to the localization of sulfanilamide in the erythrocytes.² Such localization had been demonstrated in human bloods³ and it is now known that a similar situation obtains in other species. One may reason, from these considerations, that sulfanilamide is not valid for the purpose suggested. However, the concept is of sufficient importance to require a more direct examination in the dog as the basis for its rejection or acceptance.

The volume of distribution of sulfanilamide, and its distribution in a series of representative tissues have been determined in the dog, using the concentration in the plasma water as the standard of reference.

Experimental. The renal pedicles were ligated in Dogs 1-5 under light nembutal anesthesia. Dog 6 was normal. Sulfanilamide was injected immediately after the ligation in 3 animals, the experiments being terminated in 2 (Dogs 1 and 2) and 24 hours (Dog 3). It was administered to Dogs 4 and 5 24 hours postoperatively. Dogs 3, 4 and 5 had recovered completely from the anesthetic and from the acute effects of the operative procedure at the time of the distribution studies. All animals were quickly exsanguinated at the termination of the experiment and the tissues immediately sampled for analysis; blood samples were taken one hour after the intravenous administration of the sulfanilamide and just prior to sacrificing the animal.

The sulfanilamide determinations⁴ were on protein-free filtrates of plasma, erythrocytes, and tissue obtained by the precipitation of protein at high dilution (1-50) with trichloroacetic acid. Small pieces of tissue were thoroughly ground with silica and trichloroacetic acid and then extracted for one hour with frequent agitation. Complete recovery of sulfanilamide added to plasma, erythrocytes and tissue was regularly obtained with these procedures. The sulfanilamide concentrations in plasma water were calculated from the plasma concentrations on analysis and the water content of plasma. No corrections were made for the nondiffusible sulfanilamide which is small in the case of dog plasma.^{5, 6}

¹ Painter, E. E., *Am. J. Physiol.*, 1940, **129**, 744.

² Peters, J. P., *Ann. Rev. Physiol.*, 1942, **4**, 89.

³ Sise, H. S., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 451.

⁴ Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

⁵ Waterhouse, A., unpublished.

⁶ Davis, B. D., *Science*, 1942, **95**, 78.

TABLE I.
The Distribution of Sulfanilamide in the Dog.

Dog No.	Wt. kg	Sulfanilamide administered, mg	Duration of exper., hr	Plasma sulfanilamide, mg per 100 g plasma water	Vol. of distribution		Ratio	Sulfanilamide concentration per 100 g wet tissue											
					Liters	% body wt		Erythrocytes	Cerebrospinal fluid (cerebrum)	Brain	Lung	Liver	Pancreas	Muscle	Nerve				
1	7.27	229.7	2	3.44	6.68	91.9	1.38	.62	.66	1.07	1.29	1.08	1.13	.76					
2	7.27	232.7	2	3.10	7.49	102.9	1.38	.59	.68	1.09	1.23	1.08	1.10	.76					
3	10.0	324.0	24	2.58	12.56	125.6	1.23	.65	.72	1.02	1.47	.88	.88	.77					
4	9.95	324.0	2	3.74	8.66	87.0	1.22	.56	.65	1.05	1.06	.93	.98	.72					
5	12.7	422.0	2	3.78	11.17	88.0	1.25	.57	.67	1.17	1.50	1.00	.97	.94					
6	12.3	333.0*	2	3.01	11.06	90.1	1.33	.67	.70	1.06	1.22	1.05	1.01	.77					
Water content of tissue													Ratio						
Wet weight of tissue																			
													.65	.78	.79	.72	.73	.76	.66

*333 mg represents the difference between the sulfanilamide injected and that excreted by the kidney in the 2-hour interval.

Results. These are summarized in Table I. The time allowed for the establishment of plasma-tissue equilibrium of sulfanilamide was 2 hours in 5 experiments and 24 hours in one. Two hours is quite adequate since the plasma concentration was essentially constant after the first hour in all the animals with ligated renal pedicles. The concentration of sulfanilamide, other than that in the plasma, has been expressed as the ratio of its concentration per 100 g of wet tissue to the concentration in plasma water. This ratio would be equal to the fraction of wet tissue which is water if the concentration of sulfanilamide in a tissue were determined solely by its water content. Data on the water content of tissues⁷ are included in the table for convenience of comparison. The data indicate that there is a significant localization of sulfanilamide in all tissues including brain substance if, as seems probable, the latter tissue is in equilibrium with a fluid with the chemical characteristics of cerebrospinal fluid.⁸

Discussion. The data are in keeping with the qualitative view that sulfanilamide is widely distributed in tissues in a fairly even manner.⁹ They are quite contrary to the concept that its distribution is dependent solely upon the distribution of water and, that it may be used as an accurate measure of the water content of the body. The agreement previously obtained¹ between the volume of distribution of sulfanilamide and the volume of distribution of urea, or, the directly measured water content of dogs appears to be the result of compensating errors. The localization of sulfanilamide in the erythrocytes yields a high value for its concentration in the fluid of reference when whole blood is used and this approximately balances a somewhat similar localization in other tissues.

Conclusion. Sulfanilamide may not be used as a measure of total water in the dog.

⁷ Brodie, B. B., unpublished.

⁸ Wallace, G. B., and Brodie, B. B., *J. Pharm. and Exp. Therap.*, 1939, **65**, 220.

⁹ Marshall, E. K., Jr., Emerson, K., and Cutting, W. C., *J. Pharm. and Exp. Therap.*, 1937, **61**, 196.

13741

Choline Deficiency in Rats of Various Ages.*

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Griffith and Wade¹ demonstrated that weanling rats receiving diets deficient in choline developed a hemorrhagic kidney disease within 2 weeks. Later it was shown that this abnormality could be prevented by methionine,² or by betaine,³ as well as by choline. Engel and Salmon⁴ developed a diet which was sufficiently depleted of choline, and of other compounds behaving like choline, to produce 100% mortality in weanling rats. The same diet when supplemented with adequate choline produced good growth. Data are presented herein to show: (1) the approximate minimum requirement of choline for the prevention of the fatal hemorrhagic disease during the first 2 weeks after weaning, and (2) the need for dietary choline for the prevention of the hemorrhagic disease throughout the rapid growth period.

Method. Weanling rats were placed on the choline-deficient diet 31 PMC⁴ at 23 days of age. The rats were individually fed the diet *ad libitum*, and 20 mcg each of thiamin, riboflavin, and pyridoxine, and 100 mcg of calcium pantothenate per day; they were fed adequate amounts of carotene and calciferol once a week. Choline was fed as indicated later. Two groups of rats were retained on the stock diet for 3 and 5 weeks respectively before being transferred to the deficient diet.

The Approximate Minimum Requirement of Choline. Preliminary trials on a few animals had indicated that the approximate daily choline chloride requirement for the prevention of the fatal hemorrhagic conditions was 5 mg. The results of further studies on the minimum requirements of choline are presented in summary form in Table I. Male and female rats from 10 different litters received choline chloride at the 4 mg level. It is of interest that 75% of the fatal cases occurred among the rats from 3 litters, and that there were

* Published with the approval of the Director of the Alabama Agricultural Experiment Station.

¹ Griffith, W. H., and Wade, N. J., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 188.

² Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, 1940, **132**, 627.

³ Griffith, W. H., and Mulford, D. J., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 657.

⁴ Engel, R. W., and Salmon, W. D., *J. Nutrition*, 1941, **22**, 109.

TABLE I.
Weight Change and Survival in 23-day-old Rats Receiving Suboptimum Levels of Choline.

No. of rats	Sex	Choline chloride per rat daily, mg	Avg initial wt, g	Avg wt change 1st wk, g	No. surviving 2 wks	Avg wt change* 2nd wk, g	Avg wt change fatal cases 2nd wk, g	Survival %
19	M	4	44	16	11	15	—10	58
15	M	5	47	16	12	12	— 5	80
21	F	4	45	14	17	15	— 7	81
12	F	5	44	15	11	17	— 5	92

*Survivors only.

4 litters represented in which no mortality occurred. The litter variation was not correlated with variation in rate of growth up to the crucial 7- to 10-day period when the hemorrhagic condition developed. Rats from 8 different litters each received 5 mg of choline chloride daily. In this experiment 3 of the 4 fatal cases occurred in rats from one litter. At both levels of choline feeding the male rats were slightly more susceptible to the hemorrhagic disease than the females, a confirmation of the results reported by Griffith,⁵ and by Engel and Salmon,⁴ when the latter workers used diets less severely deficient in choline and in substances behaving like choline.

In view of individual variation, as well as litter variation, it is difficult to establish any exact minimum requirement of choline for the prevention of the fatal hemorrhagic disease. The data suggest that approximately 5 mg of choline chloride per rat daily are necessary for the prevention of the fatal hemorrhagic condition in most cases. Griffith⁶ has reported that for diets containing 18 to 24% of casein and 6% of yeast, 1 to 2 mg of choline chloride per rat daily are needed to prevent kidney hemorrhage. If the methionine supplied by this amount of casein and the choline supplied by this amount of yeast are taken into account, the results reported by Griffith are confirmed in the present experiments.

The Need for Choline Throughout the Rapid Growth Period. Since relatively large quantities of choline were required by the weanling rat for the prevention of the hemorrhagic condition during the first 2 weeks it was important to determine whether this condition could be produced if the rats were supplied with choline through the crucial 2-week period. To this end, weanling rats were fed diet 31 PMC and the usual supplements plus 20 mg of choline chloride per rat daily for 2, 3, 4, and 5 weeks respectively. After these

⁵ Griffith, W. H., *J. Nutrition*, 1940, **19**, 437.

⁶ Griffith, W. H., *J. Nutrition*, 1941, **22**, 239.

periods had elapsed the rats were continued on the same diet without choline and observations were made daily. The results definitely indicate that dietary choline is essential throughout the rapid growing period.

When 20 mg of choline chloride was fed for 2 weeks after weaning and the choline then discontinued, 3 out of 4 rats died with hemorrhagic kidneys between the ninth and the twelfth day. When the choline was fed for 3 weeks, 2 out of 4 rats died, one on the seventh and the other on the fourteenth day after choline was discontinued. Of the 12 rats fed choline for 4 weeks, 6 died of choline deficiency within the succeeding 14-day period, the mean number of days to exitus being 9. Three out of 7 rats which had received choline for 5 weeks died of choline deficiency within 14 days after choline feeding was discontinued.

The results indicate that the susceptibility of growing rats of either sex to choline-deficiency hemorrhagic disease remains practically unchanged between the fourth and the seventh week after weaning. In general, the rats which received choline over longer periods (4 or 5 weeks) usually suffered the greatest weight losses before death. In all but one of these fatal cases the body weight at death was less than it was when choline feeding was discontinued. This is particularly significant since such severe weight losses did not occur in choline-deficient rats during the crucial 7- to 10-day post-weaning period.

The surviving rats were continued on the choline-deficient diet for from 4 to 12 weeks. Their weight gains were very irregular. Eight of the 13 survivors had suffered weight losses during the 2-week period following the discontinuation of choline feeding and showed connective tissue scarring of the kidney cortex at necropsy, an indication that hemorrhage had occurred.

From the previous experiments it was apparent that if rats receiving diets low in choline were deprived of choline at any time during the rapid growing period, they were susceptible to the hemorrhagic kidney disease. It seemed desirable to determine what the result would be if rats were retained on the stock diet until varying weight levels had been reached before they were placed on the choline-deficient diet.

One group of 8 rats was kept on the stock diet for 3 weeks after weaning, and another group of 9 rats was kept on the same diet for 5 weeks. All of these rats were placed on the choline-deficient diet and received the usual vitamin supplements but no choline. Four of the 8 rats which had received the stock diet for 3 weeks after weaning had lost weight after 2 weeks on the choline-deficient diet. All of

these rats survived through the first 2 weeks and resumed growth during the third week. At autopsy 3 weeks later, one of the rats which had lost weight showed severe connective tissue scarring of the kidney surface.

Two of the rats which had received the stock diet for a 5-week period before being placed on the deficient diet lost weight during the second week of the experiment and both rats had connective tissue kidney scars at necropsy.

The data suggest that rats which were fed the stock diet during the preliminary feeding trial were somewhat less susceptible to choline-deficiency disease than those which were fed the choline-deficient diet supplemented with relatively high levels of choline.

Summary. Weanling rats receiving a diet severely deficient in choline required approximately 5 mg of choline chloride per rat daily to prevent the fatal hemorrhagic kidney disease. Choline deficiency was produced within 7 to 14 days at any time during the rapid growing period when choline was omitted from the diet. Weanling rats fed a stock diet for periods of 3 or 5 weeks were somewhat less susceptible to choline deficiency than rats fed the diet low in choline and supplemented with 20 mg of choline chloride daily for similar periods.

13742 P

Alterations in X-Ray Diffraction Pattern of Rat Tibia in Rickets.*

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The use of diffractograms in the study of molecular structure has been discussed extensively by Clark.¹ Numerous studies on bone by this technic have confirmed that there is a characteristic pattern most nearly agreeing with the type pattern of dahlite. Not many reports have given consideration to the possibility that this characteristic pattern may undergo physiological alteration. Roseberry, Hastings and Morse² described briefly some age changes in cultures of osseous

* A part of the expenses of this investigation were borne by a grant from the Nutrition Research Laboratories, part by a grant from the Graduate School Research Fund.

¹ Clark, G. L., *Applied X-Rays*, 3rd, New York, 1940, McGraw-Hill Book Co.

² Roseberry, H. H., Hastings, A. Baird, and Morse, J. K., *J. Biol. Chem.*, 1931, **90**, 395.

tissue. Clark and Mrgudich³ described characteristic changes in rickets, quantitatively related to the degree of severity.

There was no fundamental change in chemical composition, since the lattice spacings were not altered. Both inorganic and organic elements were disorganized as evidenced by the much darker central area with its sharply defined boundaries and by the extension of the orientation arcs, sometimes forming a complete circle.

Several other reports have been made of qualitative alterations in diffraction patterns of bones under various experimental conditions.⁴

In an effort to study the influence of healed rickets on the subsequent behavior of bone, our laboratory first made a study of mechanical efficiency, already reported.⁵ A summary of findings in the first 5 series of those experiments is presented in Table I. From this it will be seen that in all groups on a rachitic diet for 2-3 weeks, there were no significant differences in mechanical efficiency as manifested by breaking stress, regardless of the duration of the recovery period. Significant differences were seen only when the period of rachitic feeding was extended to 6 weeks.

These same bones were used in the present study. Sections of cortex were ground from the tibial mid-shaft adjacent to the point of fracture. These were reduced to a thickness of 0.05 to 0.1 mm and mounted on a Hayes diffraction unit before a pinhole 0.025 inch in diameter. Exposure was made at 40 mm film distance to a Cu-target, G. E. X-ray tube operating at 35 kv, 22 ma for 2½ hours.

Analysis of over 500 diffractograms shows that in any long bone the most striking evidence of orientation is found in one large ring

TABLE I.
Comparison of Mechanical Resistance of Rat Tibiæ.

Series	Age on rachitic diet	Days on diet	Days on healing diet	Age killed, days	
I	25	20	65	110	Not significant
II	34	41	42	117	Significant
III	24	19	72	115	Not significant
IV	24	18	275	317	" "
V	23	37	42	102	Significant

In the last column is indicated the difference in mechanical efficiency between control and experimental animals, as determined by breaking stress.

³ Clark, G. L., and Mrgudich, J. N., *Am. J. Physiol.*, 1934, **108**, 74.

⁴ Clark, G. L., Bucher, C. S., and Lorenz, O., *Radiology*, 1931, **17**, 482; Henschen, C., *Schweiz. Med. Woch.*, 1937, **67**, 153, 182; **202**, 223; Reynolds, L., Corrigan, K. E., Hayden, H. S., Maey, I. G., and Hunscher, H. A., *Am. J. Roentgenol. Rad. Ther.*, 1938, **39**, 103, 286.

⁵ Schiller, A. A., and Struck, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 198.

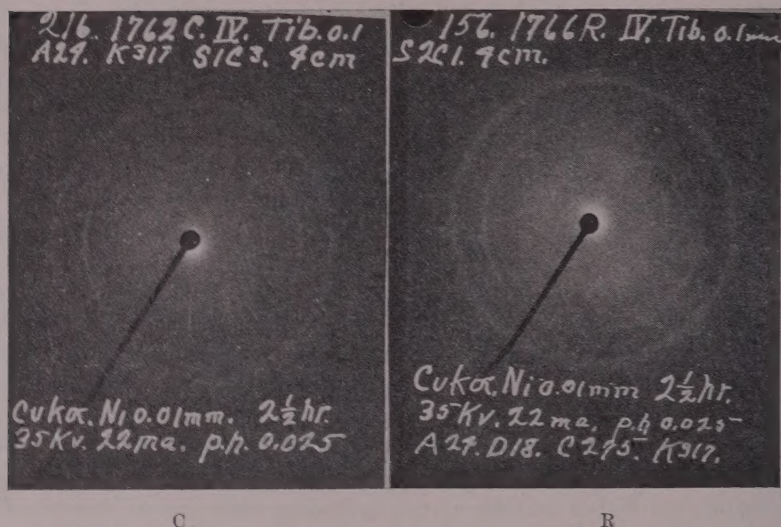


FIG. 1.

with a diameter of 40 mm and a d value 3.34 Å. This agrees with all previous observations. This ring was sharply arced in the long axis of the bone. The mean total length of the two arcs for all of the control animals at the time of sacrifice was 231° as compared to a mean of 340° for the experimental animals. This last computation was made without regard to the period on the healing diet.

A comparison of two films is presented in Fig. 1. *C* was taken from a control rat in series IV and *R* from an experimental animal in the same series. Despite the fact that this series was on a good healing diet for 275 days the rachitic pattern is clearly evident. Table I shows that mechanical efficiency of the bones in this series was not impaired. It is not clear whether this disorientation is a physiological handicap in any other respect. The point will be investigated by some other technic.

The age of this particular group at death would be comparable to 35 years for the human.

If these findings apply to the human it must be concluded that the rachitic pattern of bone is never eradicated completely. Whether its persistence has any bearing on the behavior of bone in later life remains to be determined.

13743

Studies Concerning the Site of Renin Formation in the Kidney.
II. Absence of Renin in Glomerular Kidney of Marine Fish.*

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In an earlier study,¹ the absence of detectable renin in the aglomerular kidney of the midshipman fish (Batrachoididae, *Porichthys notatus*) and its presence in the glomerular kidney of the catfish (Ameiuridae, *Ameiurus nebulosus*) and the carp (Cyprinidae, *Cyprinus carpio*) suggested to us the possibility that the formation of renin in a kidney was dependent upon its glomerular or arterial component. The midshipman fish, however, differs from the catfish and carp in that it is a marine variety of fish, whereas the latter species exist only in fresh water. Since the tubular structure of the kidney of the fresh water fish is known to differ from that of the marine fish,² it was possible that the absence of renin in the kidney of the midshipman fish was due not to the absence of glomeruli, but to a difference in its tubular properties. For this reason, it was considered advisable to examine the kidneys of other marine fish which had both glomeruli and tubules, in order to determine whether the absence of renin noted in the kidney of the midshipman fish was due to the specific absence of a glomerular apparatus or to a type of tubular development common to the kidneys of marine fish whether glomerular or aglomerular.

In the following report, the renin content of the kidneys of 2 marine fish, the sole and cod, is compared with the amount found in the kidneys of 2 fresh water fish, the carp and catfish.

All kidneys used in the study were obtained from freshly caught fish and their renin extracted in the manner previously described.¹ In most of the experiments, the extraction of renin from the dehydrated and defatted kidney powders was carried only to the stage of a crude saline extract, although in one experiment, 35 g of cod kidney were further purified and concentrated by carrying the process to the stage of "Fraction B" of Helmer and Page.³ The physiological assay of the extracts was performed as previously de-

* Aided by a grant from the Dazian Foundation for Medical Research.

¹ Friedman, M., and Kaplan, A., *J. Exp. Med.*, 1942, **75**, 127.

² Marshall, E. K., *Physiol. Rev.*, 1930, **14**, 133.

³ Helmer, O. M., and Page, I. H., *J. Biol. Chem.*, 1939, **127**, 757.

scribed.¹ Seven anesthetized (pentobarbital sodium) dogs were used, 2 of which had been nephrectomized 24 hours previously.

As reported before,¹ the carp and catfish kidney have numerous large and well lobulated glomeruli and tubular epithelium equipped with a brush border. An occasional concentration of agranular cells at the entrance of the arteriole into the glomerulus was seen, but there was no evidence of secretory action in these cells.

Histological examination of the kidney of the sole and the cod, employing both the hematoxylin-eosin and the Mallory's triple staining methods, revealed no striking difference in their respective tubular epithelium as compared to the tubular epithelium of either of the fresh water fish. However, the glomeruli of both the sole and the cod were about half the size of those found in the kidneys of the catfish and carp and also were considerably less numerous. This last observation previously had been recorded by Marshall and Smith.⁴ The kidneys

TABLE I.
Pressor Effect of Extracts from Kidney of Various Fish.

Fish kidney extr.	Dog No.	Extr. administered (g fresh kidney)	Max. rise in blood pressure* (Mm of Hg)	Avg pressure rise per g fresh kidney in extr. (Mm of Hg)
		Cod.		
A	1	4.3	-10	0.0
A	2	10.3	+ 5	0.05
B	2	5.5	0	0.0
B	1	6.6	-15	0.0
C†	3	35.0	+ 3	0.09
Avg		12.3		0.03
		Sole.		
A	2	12.2	-10	0.0
A	3	8.1	+ 5	0.62
Avg				0.3
		Catfish.		
A	7	5.0	+20	4.0
A	8	10.0	+40	4.0
Avg		7.5		4.0
		Carp.		
A	2‡	9.9	+88	8.7
A	3‡	4.5	+30	6.6
B	5	8.0	+28	3.5
C	5	20.0	+20	1.0
D	6	8.0	+48	6.0
E	1	8.0	+24	3.0
E	1	8.0	+26	3.3
Avg		9.5		4.6

*Maximum pressure rise taken 2 to 4 minutes after injection of renal extracts.

†Extraction of kidney tissue carried to Fraction B stage.

‡Recipient dog nephrectomized (24 hours).

⁴ Marshall, E. K., Jr., and Smith, H. W., *Biol. Bull.*, 1930, **59**, 135.

of the teleosts also showed an occasional concentration of agranular cells at the entrance of the arteriole into the glomerulus.

The extracts of both the carp and catfish kidney were found to contain a substance which was strongly pressor. Thus, the average rise in the pressure of dogs receiving an extract equivalent to one gram of fresh tissue was 4.6 mm of Hg for carp kidney and 4.0 mm of Hg for catfish kidney (Table I). An extract obtained from an equivalent amount of fresh hog kidney cortex effected a rise of 12 mm of Hg. Further, it was found that the pressor substance in the carp and catfish kidney had a prolonged effect, was effective in a cocainized dog, and was ineffective in a dog previously rendered tachyphylactic to hog renin. In view of these observations, it was concluded that the substance studied was renin.

In contrast to the above findings, similar assays conducted with extracts obtained from the glomerular kidneys of the cod and sole resulted in an extract which had no pressor effect upon the dog. In 5 assays of cod kidney extract, no pressor effect was observed in any of them (Table I), despite the fact that in one assay, the equivalent of 35 g of fresh cod kidney was given. Injections of 2 extracts of sole kidney, likewise failed to exert a pressor effect upon the dog.

Discussion. The apparent absence of renin in the kidneys of the cod, the sole and the midshipman,¹ as judged by the failure of their renal extracts to increase the blood pressure of nephrectomized dogs, contrasted strongly with its abundant presence in the kidneys of the fresh water fish studied. Recently, Bean,⁵ too, has reported the absence of renin in the kidney of another species of marine fish, namely, the shark. Although histological examination indicated that the kidneys of the carp and catfish had glomeruli which were both larger and more numerous than those in the kidneys of the cod and sole, this observation did not necessarily explain the apparent complete absence of renin in the latter kidneys. For it was found that whereas the extract obtained from as little as 4.5 g of carp kidney raised the dog's pressure 30 mm of Hg, an extract of 35 g of cod kidney effected no significant change in the pressure of the test animal.

Summary. The absence of renin in the aglomerular kidney of the midshipman, a marine fish, cannot now be construed as suggestive evidence of the probable formation of renin by the glomerular or arterial component of a kidney because the glomerular kidneys of other marine fish studied, also lack renin. The presence of renin in the glomerular kidneys of the catfish and carp, however, suggests

⁵ Bean, J. W., *Fed. Proc.*, 1942, **1**, 6.

that the kidney of most fresh water fish contains renin. Just why the kidney of marine fish, whether glomerular or aglomerular, should be devoid of detectable renin and the kidney of fresh water fish richly supplied with this same substance is a question which cannot be answered at this time. As has been noted, already, there is evidence that the tubular function in the kidneys of these two varieties of fish is probably not identical.

13744

Temperature Characteristics for Respiration in the Newt
Under Chloretone and Nembutal* Anesthesia.

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It is well known that various narcotics and anesthetics have a depressing effect upon respiration but this has not been systematically investigated as a function of temperature. Thus, while something is known of the influence of this class of drugs on the absolute respiratory rate, little information is available for changes in the relative respiratory rate.

The newt, *Triturus viridescens*, was used in the present experiments both because it is a poikilothermous animal and therefore useful for temperature analyses and because its respiration has already been investigated by means of a Warburg apparatus suitably modified for this purpose.

The minimum dose required to maintain newts immobile for 24 hours under the conditions of temperature variations was determined experimentally for two suitable drugs. After preliminary anesthesia for 15 minutes in a 1-10 dilution of a saturated aqueous solution of chloretone, animals were transferred to modified Warburg vessels containing 1 cc of a 1-20 dilution of a saturated solution of chloretone. Comparable fluid was introduced into one unit serving as a thermobarometer. Nembutal (sodium pentobarbital) produced the desired effect with an intraperitoneal injection of 0.2 cc of a 0.6% solution.

Thermal adaptation and the procedure for measuring oxygen consumption followed that described by Pomerat and Zarrow.¹

* Supplied through the courtesy of the Abbott Laboratories.

¹ Pomerat, C. M., and Zarrow, M. X., *J. Cell. and Comp. Physiol.*, 1937, **9**, 397.

All data reported were based on animals which remained immobile during the entire period of measurement and which recovered from the anesthetic at its conclusion.

Arrhenius plots of the data for oxygen consumption during chloretone anesthesia yielded a temperature characteristic (μ) of $17,200 \pm$ (Fig. 1). This is the same value as that found for unanesthetized animals by Pomerat and Zarrow¹ and for newts studied in relation to their endocrine system by Pomerat.² A similar treatment of the values obtained for 10 different animals under nembutal anesthesia is represented in Fig. 2. A distinct shift in the μ is seen to have occurred. A value of $22,080 \pm$ was found for temperatures from 6.5°C to 16.5°C and of $10,100 \pm$ for the temperature range of 17.0°C to 21.0°C . A critical temperature (break) was found at approximately 16.5°C . It is noteworthy that the three values for μ which have been found for the newt are commonly associated with processes in which oxygen is utilized.

Shifts in temperature characteristics such as that resulting from nembutal anesthesia are relatively rare in the literature.³ They may be looked upon simply as a modification of a fundamental constant, but from a more speculative standpoint^{3, 4, 5} they are believed to represent a change of "pace-setter" in a catenary

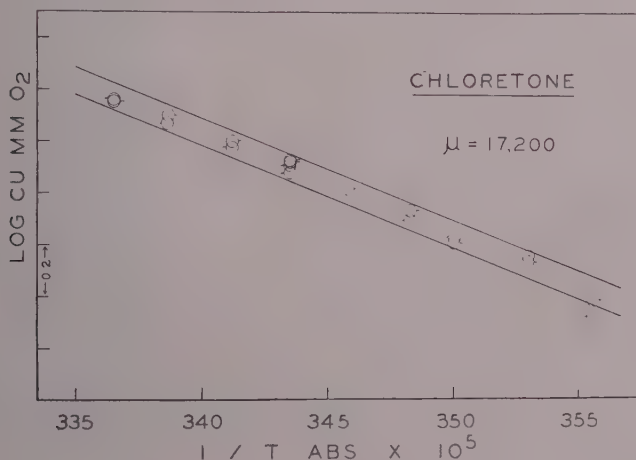


FIG. 1.

Arrhenius equation plot for the rate of oxygen consumption in the newt, *Triturus viridescens*, under chloretone anesthesia.

² Pomerat, C. M., *Endocr.*, 1939, **25**, 385.

³ Crozier, W. J., *J. Gen. Physiol.*, 1935, **18**, 801.

⁴ Crozier, W. J., and Hoagland, H., *Handbk. of Gen. Exp. Psych.*, 1934, Chap. 1.

⁵ Hoagland, H., *Cold Spring Harbor Symp. on Quant. Biol.*, 1936, **4**, 267.

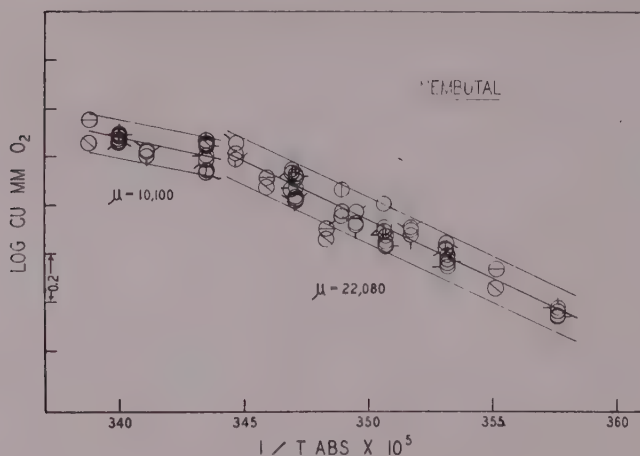


FIG. 2.

Temperature characteristics for the rate of oxygen consumption in the newt, *Triturus viridescens*, under the influence of nembutal anesthesia.

series. Among the recent studies supporting this interpretation are Hadidian and Hoagland's^{6, 7} findings concerning a respiratory enzyme system extracted from beef heart. Following poisoning with a critical amount of NaCN the value of μ shifted abruptly from $11,200 \pm 200$ to exactly 16,000 calories. This is believed to represent a change from a "pace-setter" mechanism involving a dehydrogenase to one characterized by an oxidase.

Promising among the various theories of narcosis is the view that the phenomenon is due to interference with oxidative mechanisms. According to Holmes⁸ oxidation is inhibited in tissues which are anesthetized by urethane and the higher alcohols and cytochrome remains in the oxidized form following the action of these agents as well as after treatment with chloroform and ether. Anesthetics and cyanide are believed to inhibit at different points. That these effects involve brain tissue has been supported by the work of Jowett and Quastel⁹ who found narcotic inhibition of glucose oxidation in the presence of brain slices *in vitro*. Jowett¹⁰ concludes upon further study of anesthetics acting upon brain slices that the mechanisms of these depressants lies in their capacity to inhibit cell oxidation.

⁶ Hadidian, Z., and Hoagland, H., *J. Gen. Physiol.*, 1940, **23**, 81.

⁷ Hadidian, Z., and Hoagland, H., *J. Gen. Physiol.*, 1941, **24**, 339.

⁸ Holmes, E., *The Metabolism of Living Tissues*, 1937, Cambridge University Press.

⁹ Jowett, M., and Quastel, J. H., *Bio. Chem. J.*, 1937, **31**, 565.

¹⁰ Jowett, M., *J. Physiol.*, 1938, **92**, 322.

Depressants of the barbiturate series are known to suppress respiration and transform mammals into poikilothermous animals.^{11, 12} In contrast to chlorotone it would seem that nembutal, a barbiturate, may bring about narcosis by shifting some enzymatic reaction whose presence can be detected by the measurement of the oxygen consumption as a function of temperature.

13745 P

Study of the Papilloma Virus Protein with the Electron Microscope.*

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Information relative to the physical character of the papilloma virus protein¹ has been obtained by means of ultracentrifugation,² electrophoresis,^{3, 4} diffusion and viscosity.² Recently, more direct studies of this animal virus have been undertaken with the electron microscope. In the present paper are described the preliminary observations dealing chiefly with data gathered with this instrument relative to the appearance and size of the virus.

Papilloma virus protein isolated in 0.05 M phosphate buffer pH 6.5 by previously described ultracentrifugal procedures¹ was diluted with water to concentrations of the order of 0.05 to 0.5 mg per cc. The salt concentration in the final solutions was approximately 0.005 M. Preparations for study were made by pipetting the solutions onto collodion membranes supported on 200-mesh wire gauze. Free fluid was removed with the pipette, and the resulting

¹¹ Herrmann, J., *J. Pharm. and Exp. Therapeutics*, 1940, **72**, 130.

¹² Hemingway, A., *Am. J. Physiol.*, 1940, **134**, 350.

* This work has been aided by grants from Lederle Laboratories, Inc., Pearl River, New York, and by the Dorothy Beard Research Fund.

¹ Beard, J. W., Bryan, W. R., and Wyckoff, R. W. G., *J. Infect. Dis.*, 1939, **65**, 43.

² Neurath, H., Cooper, G. R., Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1941, **140**, 293.

³ Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1942, **142**, 193.

⁴ Sharp, D. G., Hebb, M. H., Taylor, A. R., and Beard, J. W., *J. Biol. Chem.*, 1942, **142**, 217.

thin film allowed to dry in the air. For comparison, examinations have been made also of mixtures of papilloma and tobacco mosaic viruses,[†] the latter in concentration of about 0.4 mg per cc of the mixture.

The essential findings are illustrated in Figs. 1 and 2. In Fig. 1 are shown the results obtained with papilloma virus alone. Repeated micrographs of 3 different batches of the purified protein have resulted consistently in observation of the circular images shown in Fig. 1. When the protein concentration was kept in the region of approximately 0.1 mg per cc, the images were for the most part single, though grouping was frequent as seen in Fig. 1. In preparations of higher concentration, large groups of particles occurred and fewer single images were seen. A regular arrangement of images in the groups was not observed. Evidence of material in the preparations other than that revealed in the circular images was remarkably slight. Since the material examined behaved initially as the papilloma virus, it appeared likely that the images seen were micrographs of the virus particles. In Fig. 2 is shown a mixture of

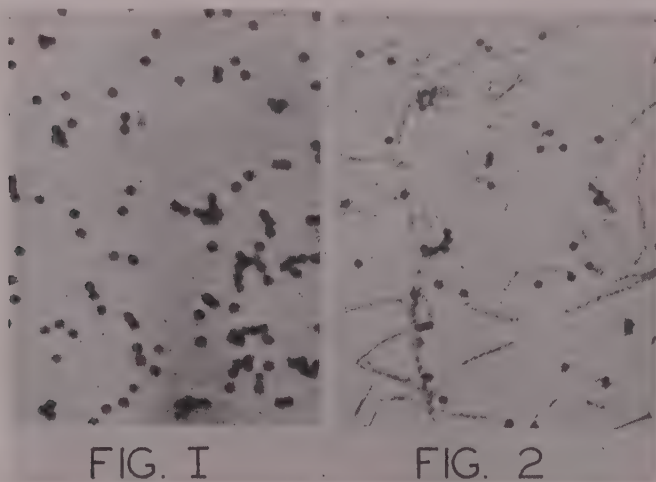


FIG. 1. Rabbit papilloma virus protein. (29,000 \times .)

FIG. 2. Mixture of rabbit papilloma virus and tobacco mosaic virus. (27,000 \times .)

These electron micrographs were made at 55 kilovolts, and the magnification in the final pictures was calculated on the basis of measurements on the tobacco mosaic virus rods which have been reported to be 15 $m\mu$ in diameter.

[†] The tobacco mosaic virus was available through the generosity of Dr. W. M. Stanley, Rockefeller Institute for Medical Research, Princeton, N.J. The virus had been purified at least 2 months prior to examination.

the papilloma virus protein with tobacco mosaic virus. The appearance of the latter was similar to that described by Stanley and Anderson.⁵ Besides the rod-shaped images, there were others which had the appearance of fragments⁵ of tobacco mosaic virus rods. These were probably not associated with the papilloma virus as they were not seen in preparations of this material in the absence of tobacco mosaic virus.

Measurements have been made of the diameter of 21 papilloma virus images in the presence of tobacco mosaic rods as in Fig. 2. Similar measurements were made also on the width of 21 tobacco mosaic rods. The values obtained for both varied rather widely. In the instance of the papilloma virus images, the standard deviation was 10.8% of the mean as compared with 12.2% for the diameter of the rods. The ratio of the mean diameter of the circular images to mean rod width was 2.93. The width of the rods of tobacco mosaic virus has been reported to be $15\text{m}\mu$ as determined both by X-ray and electron microscope.⁵ On this basis the mean diameter of the papilloma virus images was $44.0\text{ m}\mu$.

From the studies thus far made the preparations of the papilloma virus protein appear to be of considerable homogeneity with respect to particles comparable in size and shape as revealed by the electron microscope. Data previously obtained² by diffusion, viscosity and sedimentation studies indicate that the papilloma virus particle behaves as if it were an oblate ellipsoid with an axial ratio of 11 if hydration is neglected. In the present work, images of the papilloma virus particle have been consistently round, a finding compatible with the results of the earlier studies. On the other hand, if the particles were actually disc-shaped, then no particles were encountered that were not lying flat in the focal plane. Only the round form has been seen in aggregates where "rouleau" formation might well have been expected though the specimens were dried. The electron microscope data are not sufficient to permit a definite estimate of the shape of the papilloma virus particle. It is of interest, however, to consider the values obtained in the light of previous findings by indirect methods. The molecular weight of the papilloma protein,² calculated from sedimentation and diffusion data is 47,100,000, and the density by pycnometer measurement is 1.32. A spherical particle of these characteristics would have a diameter of $48.2\text{ m}\mu$, a value in good agreement with that of $44.0\text{ m}\mu$ found in the present work. A flat ellipsoid of the same weight and density would have a major axis considerably greater than 44.0 .

⁵ Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, 1941, **139**, 325.

Studies on Histamine Sensitivity and Anaphylactic Response. The Effect of Thyroid Extract.

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Administration of large amounts of thyroid extract enhances the anaphylactic response of guinea pigs.¹ It has further been demonstrated in this species that administration of thyroid extract (or of thyroxine) depletes the ascorbic acid² and cholesterol stores³ of the adrenals. Depletion of the ascorbic acid content of the adrenals in guinea pigs in turn leads to depletion of their "cortin" content.⁴

Cortin deficiency, in our opinion, leads to increased histamine sensitivity and thereby to enhanced anaphylactic response. On the basis of these considerations we would expect that administration of thyroid extract leads to increased histamine sensitivity. The following experiments were undertaken to ascertain whether this assumption is correct.

Experimental. Young adult male guinea pigs weighing on an average 350 g were used. They were fed a diet consisting of oats, carrots and greens. As there possibly exists a seasonal variation in the guinea pig's sensitivity to histamine, control experiments and thyroid experiments were done during the same season.

I. Experiments in guinea pigs fed thyroid extract. 1. Control Experiments. Forty-four guinea pigs were used. They were divided into 2 groups consisting of 18 and 26 animals. The first group received 0.3 mg histamine phosphate per kilo of body weight, the second group 0.35 mg histamine phosphate. The histamine injections were given intravenously (jugular vein) 18 to 24 hours after the last feeding. In the first group, 2 animals or 11.1% and in the second group, 9 animals or 34.6% died in shock.

2. Administration of Thyroid Extract. a. In these experiments, 36 guinea pigs, which were divided into 2 groups of 21 and 15 ani-

¹ Képinow, L., *Compt. rend. Soc. de biol.*, 1915, **78**, 198.

² Demole, V., and Ippen, F., *Z. f. physiol. Chem.*, 1935, **235**, 226; Mosonyi, J., *Ibid.*, 1935, **237**, 173; Plaut, F., and Bülow, M., *Klin. Wchnschr.*, 1935, **14**, 1318; Nespor, E., *Compt. rend. Soc. de biol.*, 1936, **122**, 427.

³ Hoen, E., Langefeld, H., and Oehme, C., *Endokrinologie*, 1939, **21**, 305.

⁴ Giroud, A., and Santa, N., *Compt. rend. Soc. de biol.*, 1939, **131**, 1176; Giroud, A., Santa, N., and Martinet, M., *Ibid.*, 1940, **134**, 23; Giroud, A., Santa, N., Martinet, M., and Bellon, M. T., *Ibid.*, 1940, **134**, 100.

imals, received orally 5 doses of 0.02 g thyroid extract U.S.P. per 100 g of body weight by mouth over a period of 5 to 6 days. Eighteen to 24 hours after the last thyroid administration and last feeding the animals received 0.3 and 0.35 mg histamine phosphate per kilo of body weight respectively. In the first group, 18 or 85.7% and in the second group 11 or 73.3% died in shock.

b. To exclude the possibility that the high percentage of deaths observed in the thyroid-fed guinea pigs was due to instability of the autonomous nervous system and not to the injected histamine, another group of 10 animals was given thyroid extract in the above described manner. Eighteen to 24 hours after the last administration of thyroid, each animal received 0.2 cc physiological saline intravenously. No reaction to this injection was observed in any animal. Twenty-five minutes later, each guinea pig received intravenously 0.3 mg histamine phosphate per kilo of body weight. Eight of the guinea pigs died in severe shock.

II. Ascorbic Acid Determinations. Various authors² have shown that administration of thyroid extract leads to depletion of the ascorbic acid stores of the tissues. Assays were done to determine the degree of depletion of ascorbic acid in the adrenals in our experiments. The method used was that of Bessey and King.⁵ The determinations were made in February and March.

The ascorbic acid content of the adrenals of 11 normal guinea pigs ranged from 64 mg % to 132 mg %. The average value was 95 mg %.

Eight guinea pigs were fed 0.02 g thyroid per 100 g of body weight 5 times over a period of 5 to 6 days. The ascorbic acid determinations were done 18 to 24 hours after the last administration of thyroid. The values found ranged from 33 mg % to 57 mg %. The average value was 47 mg %, a marked decrease.

Conclusions. In our experiments, administration of thyroid extract to normal guinea pigs greatly enhanced their sensitivity to histamine shock. We believe that the enhanced sensitivity to histamine which follows administration of thyroid extract explains the enhanced anaphylactic response observed after administration of this hormone.

Administration of thyroid extract to normal guinea pigs led to marked decrease of the ascorbic acid content of the adrenals. Depletion of the ascorbic acid stores of the adrenals of guinea pigs leads to depletion of their cholesterol and cortin contents. We suggest that the enhanced sensitivity to histamine observed in our guinea pigs is due to depletion of the cortin content of their adrenals.

⁵ Bessey, O. A., and King, C. G., *J. Biol. Chem.*, 1933, **103**, 687.

Yeast Extracts to Overcome Depressant Effects of Germicide on Skin Respiration.

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It is well known that the use of bactericidal agents in wounds is hampered by the adverse effects of these agents upon the tissues. For example, Bisgard and Baker "believe that there is ample experimental and clinical evidence that the chemical bactericidal agents, which are so generally used to 'disinfect' wounds, destroy or devitalize the tissue cells with which they come in contact and thereby seriously impede the inflammatory response and impair the effectiveness of the natural mechanism of defense."¹

It is obvious that an agent which would, even in part, offset the deleterious effects of bactericides on tissues with preservation of adequate bactericidal powers might be of clinical value.

In the course of studies on the effects of yeast and tissue extracts on cellular metabolism we observed that certain yeast extracts antagonized the toxicity of phenylmercuric nitrate and *n*-butyl-*p*-hydroxybenzoate for molds.² It appeared to us that such extracts, and particularly certain fractions known to increase tissue respiration,³ might serve to protect the tissues from the toxic action of germicides.

As a measure of toxicity to tissues we used the respiratory-depressing effect of the germicide since respiration is a satisfactory index of tissue vitality⁴ and since a number of workers have proposed a manometric method of germicide evaluation based upon the correlation of the toxic effects of germicides with their respiratory depressant activities on organism and host tissue.⁵ In line

¹ Bisgard, J. D., and Baker, C. F., *Surg. Gynecol. Obstet.*, 1942, **74**, 20.

² Cook, E. S., and Kreeke, C. W., *Nature*, 1940, **146**, 688.

³ Cook, E. S., Kreeke, C. W., and Nutini, L. G., *Studies Inst. Divi Thomae*, 1938, **2**, 28.

⁴ Adams, P. D., *Arch. Dermatol. and Syphilol.*, 1936, **36**, 606; Cook, E. S., *Chem. Products*, 1939, **1**, 65, and 1939, **2**, 89; Amersbach, J. C., Nutini, L. G., and Cook, E. S., *Arch. Dermatol. Syphilol.*, 1941, **43**, 949; Manifold, M. C., *Proc. Roy. Soc. Med.*, 1940, **33**, 12.

⁵ Braufenbrenner, J., Hershey, A. D., and Doubly, J., *J. Bact.*, 1939, **37**, 583; Ely, J. D., *Ibid.*, 1939, **35**, 391; Greig, M. E., and Hoogerheide, J. C., *Ibid.*, 1941, **41**, 549, 557; Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **73**, 749.

with these findings, we had also noted that, with certain fractions from yeast, ability to stimulate yeast respiration paralleled ability to increase yeast proliferation.⁶

The experiments reported in the present paper concern the toxic effects of phenylmercuric nitrate on rat skin as observed by manometric determination of skin respiration, the overcoming of these toxic effects by the addition of a yeast extract, and a comparison of the germicidal efficiency of the drug on *Staphylococcus aureus* with and without the addition of the extract.

Respiration measurements were made by the previously described⁸ Warburg method on the abdominal skin of female albino rats approximately one year old. Determinations were made at 37.5°C in Ringer-phosphate-glucose solution (0.2% glucose, pH 7.2). Aqueous-alcoholic yeast extracts were prepared as described previously⁸ from Fleischmann's bakers' yeast and corresponded to Fraction A of the earlier work.⁸ A stock culture of *S. aureus* obtained from our bacteriology laboratory was grown in nutrient broth. Commercial basic phenylmercuric nitrate (Merphenyl Nitrate, Hamilton Laboratories) was employed.

Fig. 1 shows a typical experiment on the effects of phenylmercuric nitrate and of a crude yeast extract upon the respiration of rat skin. Each point represents the average of 3 determinations, which agreed within 10%, on skin from the same animal. Curve I represents the respiration of rat skin under the experimental conditions. In Curve II skin and phenylmercuric nitrate (1:100,000 final concentration) were present at the beginning of the experiment. The toxic effects of the germicide are evident, and at the end of 60 minutes the skin was respiring 24% below the control. At this time, yeast extract was tipped from the side arm to give a concentration of 10 mg of solids per cc (1%). An increase in respiration rate is apparent and the slope of the remainder of Curve II approaches that of Curve I. In Curve III skin and yeast extracts were present at the beginning of the experiment. At the end of 60 minutes a 48% stimulation of skin respiration exists. Addition of phenylmercuric nitrate at this time reduces the respiratory rate to that of the control, with the net result that the respiratory depressant effect of the germicide is offset.

In another set of 3 determinations, doubling the concentration of germicide (1:50,000) doubled the inhibition of skin respiration (46% inhibition as compared with 24% above). Addition of 1% of yeast extract at the end of 60 minutes was ineffective in off-

⁶ Cook, E. S., Hart, M. J., and Stimson, M. M., *Biochem. J.*, 1940, **34**, 1580.

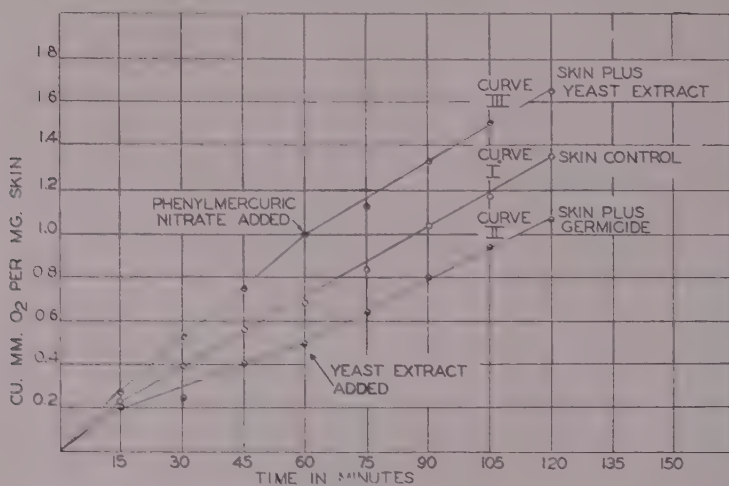


FIG. 1.

Effects of phenylmercuric nitrate and yeast extract on respiration of rat skin.

setting the germicidal depression, but the addition of the yeast extract one hour prior to the addition of the germicide reduced the inhibitory effect of the latter from 46% to 20%. Thus, this concentration of yeast extract did not overcome the greater depression previously caused by a higher concentration of germicide but it did afford protection when added in advance of the germicide. Addition of a mixture of germicide (1:100,000) and yeast extract (1%) after 60 minutes had no effect on the respiratory rate, suggesting either that the inactivation occurs by some sort of combination between the active substances or that the active ingredient of the extract reaches the respiratory systems of the cell at least equally as readily as the germicide.

Repetition of the experiments with different yeast extracts gave results which accorded qualitatively with the above and differed only in amounts of stimulation or depression. This would be expected with the use of unstandardized preparations. A total of 38 determinations was made.

Entirely similar experiments on the respiratory antagonism between phenylmercuric nitrate and yeast extract have been carried out using yeast in place of skin, and the relation between respiratory depression and toxicity has been developed by means of methylene blue staining and parallel experiments on the effects of germicide and yeast extract on growth of yeast in liquid and solid media. These experiments will be reported elsewhere.

In order to see whether the overcoming of toxicity of phenyl-

mercuric nitrate for skin by the yeast extract was accompanied by a loss of toxicity for a typical infectious organism, the following experiment was performed in duplicate. Two sets of tubes each containing 10 cc of nutrient broth were inoculated with a loop of a 3-day broth culture of *Staphylococcus aureus*. In one set of tubes were incorporated dilutions of phenylmercuric nitrate and in the other were incorporated the same dilutions of phenylmercuric nitrate plus 1% (by weight) of yeast extract. After 24 hours' incubation at 37.5°C the amount of growth was determined by centrifugation in Hopkins tubes. Results of a typical experiment are shown in Table I. The yeast extract used for this experiment was the same as that employed in the respiration experiment of Fig. 1. It is readily seen that the yeast extract increases the growth of *S. aureus* in broth and allows growth in dilutions of phenylmercuric nitrate up to 1:1,000,000 which are otherwise germicidal. However, in dilutions of germicide of 1:500,000 or less no growth occurs even in the presence of 1% of yeast extract. Similar results were obtained when *S. aureus* was grown on nutrient agar and the amount of growth was determined by planimeter measurements of the colonies. Results on the solid medium showed somewhat greater variation than those obtained in-broth, the latter checking almost exactly.

From the experimental results above it is evident that the toxicity for skin of dilutions of phenylmercuric nitrate as low as 1:100,000 can be offset by 1% of yeast extract. Such dilutions of germicide in the presence of 1% of yeast extract are still completely effective against *S. aureus*. Thus, it should be possible to maintain germicidal efficiency while minimizing toxic action on the host tissue. Preliminary experiments have indicated that combinations of phenylmercuric nitrate and yeast extract also retain germicidal power against *E. coli* and a hemolytic strain of *S. aureus* when tested by the cup plate method, and similar combinations permit the growth of chick epithelium in tissue culture. An extension of these studies

TABLE I.
Effect of Phenylmercuric Nitrate and Yeast Extract on Growth of *S. aureus*.

Dilution of phenylmercuric nitrate	Growth in cc sediment	
	No extract	1% extract
1:200,000	0	0
1:300,000	0	0
1:400,000	0	0
1:500,000	0	0
1:1,000,000	0	.001
1:2,000,000	0	.001
No phenylmercuric nitrate	.005	.007

together with clinical investigations are now in progress.

Summary. One percent of an aqueous-alcoholic yeast extract protects rat skin against the toxic effects of phenylmercuric nitrate in a dilution of 1:100,000 as determined by respiration measurements. A similar amount of yeast extract does not impair the germicidal efficiency of phenylmercuric nitrate against *S. aureus* in dilutions as low as 1:500,000 under the test conditions. Protection of host tissue against toxic action of germicides with maintenance of germicidal properties is thus suggested.

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Failure of Yellow O.B. to Produce Neoplasms.

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Both butter-yellow (*p*-dimethylaminoazobenzene) and *o*-aminoazotoluene are known to be highly effective in producing liver cancer in rats.¹ Since the former dye has been used to color oils, oleomargarine, and other vegetable fat substitutes for butter, and Yellow O.B. (1-*o*-tolylazo-2-naphthylamine), and Yellow A.B. (1-phenylazo-2-naphthylamine), oil-soluble coal tar dyes are permitted in this country to color foodstuffs, the possibility of their acting in an analogous manner to butter-yellow cannot be overlooked as these substances are somewhat related in structure to butter-yellow and *o*-aminoazotoluene (Fig. 1).

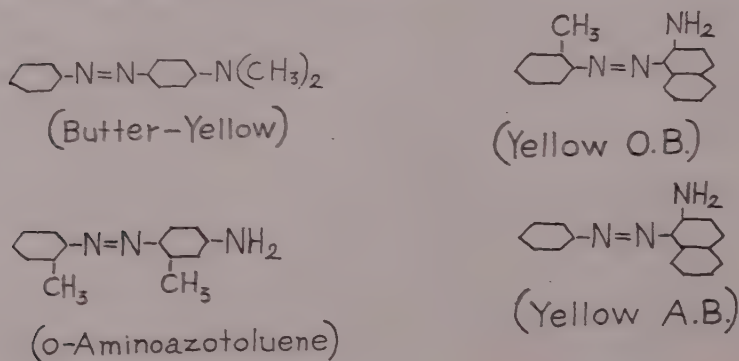


FIG. 1.

¹ Sugiura, K., and Rhoads, C. P., *Cancer Research*, 1941, **1**, 3.

In view of this fact, it seems desirable to determine whether or not ingestion of large amounts of these oil-soluble coal tar dyes would lead to production of liver cancer.

Experimental. In the present series of experiments Yellow O.B. (National Aniline and Chemical Co.) was tested first because it is more commonly used than Yellow A.B. This substance was dissolved in olive oil in the proportion of 6%. Twenty cc of this solution was mixed with 1000 g of coarsely ground, unpolished Texas rice. The basal diet was supplemented with a small slice (about 1 g) of fresh carrot per rat daily. Unlimited water was allowed. Thirty-one young adult rats (about 125 g body weight) of the Sherman stock were used.

The results showed that the daily ingestion of large amounts of Yellow O.B. (each animal consumed 7 to 12 mg of the dye daily) failed to produce liver cirrhosis or tumors in rats during 78 to 259 days' feeding.

The livers of animals fed the Yellow O.B.-rice diet were very pale and yellowish in color. There was no great change in their size or shape. The livers had smooth surfaces and histological examination showed no evidence of tumors, bile duct changes, or abnormal regeneration of the ducts and liver cells, and no abnormal nuclear changes. However, there was a lesion consisting of a zone of central necrosis about the efferent vein involving a maximum of about one-third of the lobule. This acute lesion was not a constant finding, and not incidental to the bronchopneumonia.

The lungs of these animals were very pale with patchy areas of redness, but the heart, kidney and spleen showed no great changes in size, color, or shape. No visceral lesions were seen except for bronchopneumonia. No tumor was found in these viscera or elsewhere.

During the first 80 days the nutrition of the experimental animals was good. During the next 70 days, the general appearance of the animals was fairly good, but in some instances they appeared undernourished and weakened. Individual animals consumed about 6 to 10 g of the basal diet. Of the 31 rats studied 23, or 74%, died between 78 and 259 days. Control rats maintained on a similar diet without Yellow O.B. supplement demonstrated a healthier appearance, and among 18 rats, 6 or 33%, died during the corresponding period of time.

Conclusion. Yellow O.B. (1-*o*-tolylazo-2-naphthylamine) is not a carcinogenic substance.

The author wishes to express his appreciation to Dr. C. P. Rhoads for his interest and valuable advice.

In vitro* Effect of α -Tocopherol Phosphate on Oxygen Consumption of Muscle from Vitamin E-deficient Animals.

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Confirming previous observations¹ on nutritionally dystrophic rabbits and rats, Houchin² recently reported the oxygen consumption of dystrophic hamster muscle to be much above that of normal muscle. It was also demonstrated that within 27 hours after administration of α -tocopherol acetate to the dystrophic hamster, the Q_{O_2} of the muscle was reduced toward normal value without significant regeneration of normal muscle status as indicated by a persistent high chloride content.

These observations naturally suggested that α -tocopherol may play a rôle in the normal processes of muscle metabolism. The data in this paper establish the fact that α -tocopherol phosphate[†] acts *in vitro* on muscle cells from vitamin E-deficient animals to lower the excessive oxygen consumption toward the normal.

Vitamin E-deficient rabbits, hamsters, and adolescent rats were studied. The oxygen consumption of muscle slices was determined in the usual manner in the Warburg apparatus. The control medium was a modified Locke-Ringer's solution; to prepare the experimental medium 5 mg α -tocopherol phosphate were dissolved in a few drops of water and made up to 100 cc with the Locke-Ringer's solution. The solution was well shaken immediately before the required amount was measured out. The results are expressed as Q_{O_2} (cubic millimeters of oxygen per milligram dry weight per hour).

As seen in Table I the oxygen consumption of muscle from E-deficient rabbits and hamsters in the medium containing α -tocopherol phosphate was 41.3% and 35.7% lower than that of slices from the same muscle in the unsupplemented medium. The oxygen consumption was thus considerably reduced toward that of normal animals.

The oxygen consumption of the muscle of control animals was

* Aided by a grant from Parke, Davis and Company.

¹ Victor, J., *Am. J. Physiol.*, 1934, **108**, 229; Madsen, L. L., *J. Nutrition*, 1936, **11**, 471; Friedman, I., and Mattill, H. A., *J. Physiol.*, 1941, **131**, 595.

² Houchin, O. B., *Proc. Fed. Am. Soc. Exp. Biol.*, 1942, **1**, 117.

[†] Kindly sent to us by Hoffman-LaRoche, Inc.

TABLE I.
The *in vitro* Effect of α -tocopherol Phosphate on the Oxygen Uptake of Muscle from Vitamin E-deficient and Normal Animals.

Animal and condition	No. of exper.	Q _{O₂} (averages)		
		Control, Locke's Sol.	Exper., Locke's Sol. containing 5 mg % α -tocopherol PO ₄	% decrease
E-Def. Rabbit	4	2.54	1.45	41.3
E-Def. Hamster	5	2.89	1.86	35.7
E-Def. Hamster, destroyed*	2	0.07 \pm	0.06 \pm	
Normal Hamster	1	1.74	1.78	-0.8
Normal Rabbit	2	1.34	1.40	-4.3
E-Def. Rabbit, treated†	2	1.72	1.62	6.2

*Tissue slices dropped in boiling Locke's solution, and heated 3 minutes.

†Given a therapeutic dose of 25 mg α -tocopherol acetate 48 hours previously.

not altered beyond experimental error by the medium containing α -tocopherol phosphate. When the Q_{O₂} had already been lowered by previous administration of α -tocopherol to dystrophic rabbits, the addition of α -tocopherol phosphate to the medium caused no significant change. The Q_{O₂} of muscle slices after immersion for 3 minutes in boiling Locke-Ringer's solution was negligible and was not influenced by α -tocopherol phosphate. The α -tocopherol phosphate thus participates in a biological system and does not act merely as a chemical antioxidant. The results with rats were less striking and the creatine content of the various muscles could not be correlated with the respiratory data. Results with α -tocopherol *in vitro* have been inconclusive; whether the emulsion secured was not sufficiently fine, or whether tocopherol requires phosphorylation to be active, are open questions.

Since α -tocopherol phosphate did not affect the Q_{O₂} of normal muscle or of muscle from dystrophic animals that had previously been given α -tocopherol, it may be assumed that these muscles already contained the optimum amount necessary for normal behavior. This would also indicate that some form of α -tocopherol acts directly in muscle enzyme systems to inhibit or regulate oxidation. Certain substituted phenols have been shown to inhibit respiration in fertilized *Arbacia* eggs and to inhibit the catalytic activity of flavoprotein,³ and of cytochrome reductase.⁴ The phenolic group in α -tocopherol may normally provide a point of delay to the progress of the oxidative process in muscle tissue. In the absence of

³ Krahle, M. E., and Clowes, G. H. A., *J. Gen. Physiol.*, 1940, **23**, 413; Krahle, M. E., Keltch, A. K., and Clowes, G. H. A., *J. Biol. Chem.*, 1940, **136**, 563.

⁴ Haas, E., Harrer, C. J., and Hogness, T. R., *J. Biol. Chem.*, 1942, **143**, 341.

α -tocopherol, oxidation would be unimpeded at this point and could continue at an excessive rate. Information on the enzyme systems concerned is necessary to an understanding of the rôle of tocopherol in muscle physiology.

13750

Plasma Volume Changes Following the Intravenous Injection of Pectin and Physiologic Saline in Man.

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The use of pectin as a blood substitute in the treatment of patients in shock has been proposed.¹ Since the rationale for the administration of pectin is to increase the plasma volume, information regarding the effect of this material upon the plasma volume is desirable. The present investigation was undertaken to determine the influence of the intravenous injection of a 0.75% solution of pectin[†] upon the plasma volume, and to compare the results with similar observations following the intravenous injection of physiologic saline.

Nine male patients, who were normal with respect to the cardiovascular system were studied. The plasma volume was determined by the method of Gibson and Evans² as modified by Gibson and Evelyn³ using "Evans Blue" dye (T-1824). In 5 patients after the initial determinations were made, pectin solution was injected intravenously at a rate of 10 cc per minute. In 2 patients dye was reinjected and the plasma volume was redetermined immediately upon completion of the pectin infusion. In 3 patients the second plasma volume determination was made 4 hours after the end of the pectin infusion. In all cases a final determination was made at the end of 24 hours. Similar determinations at corresponding time

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¹ Hartman, F. W., Schelling, V., Harkins, H. N., and Brush, B., *Ann. Surg.*, 1941, **114**, 212.

[†] We are grateful to Dr. Frank W. Hartman of the Henry Ford Hospital, Detroit, Mich., for his coöperation in supplying us with the pectin solution used in these experiments.

² Gibson, J. G., and Evans, W. A., *J. Clin. Invest.*, 1937, **16**, 301.

³ Gibson, J. G., and Evelyn, K. A., *J. Clin. Invest.*, 1938, **17**, 153.

intervals were made in 4 patients following the intravenous injection of 1000 cc of physiologic saline.

Results. Table I shows the results obtained following the injection of an average of 690 cc of pectin in 5 patients as compared with the results in 4 patients each of whom received an intravenous injection of 1000 cc of physiologic saline. In the first 2 cases the plasma volume immediately following the injection of pectin was increased by an amount corresponding to the volume of pectin solution injected. In the next 3 cases the plasma volume 4 hours after the end of the pectin injection was significantly increased, the amount of increase being greater than the volume of the pectin solution injected. At the end of 24 hours the plasma volume was approximately its initial value in 2 patients (J.P., R.M.) while in 3 patients (L.C., J.R., E.W.) a definite increase in plasma volume was still present.

Plasma volume determinations done at corresponding time intervals following the injection of 1000 cc of physiologic saline indicate that immediately after the end of the injection the amount of increase in plasma volume was significantly less than the volume of fluid injected. At the end of 4 hours following the 1000 cc infusion the average increase in plasma volume was only 350 cc. At the end of 24 hours the plasma volume was approximately the initial level.

Discussion. We have determined the alterations in plasma volume following the intravenous injection of 0.75% pectin solution and physiologic saline in 9 men with no cardiovascular disease. The

TABLE I.
Comparison of Plasma Volume Changes Following Injection of Pectin and Physiologic Saline.

Patient	Initial plasma vol., cc	Amt injected cc	P.V. in cc at end of infusion	Increase or decrease cc	P.V. in cc 4 hrs after end of infusion	Increase or decrease cc	P.V. in cc 24 hrs after end of infusion	Increase or decrease cc
<i>Pectin</i>								
J.P.	2760	750	3540	+780			2800	+ 40
L.C.	2700	675	3300	+600			2940	+240
J.R.	2710	750			3630	+920	2830	+120
E.W.	3150	625			3820	+670	3580	+430
R.M.	3730	650			4700	+970	3720	- 10
Avg		689		+690		+853		+164
<i>Saline</i>								
R.P.	3490	1000	3980	+490			3540	+ 50
R.K.	3025	1000	3340	+315			3200	+175
F.D.	3010	1000			3340	+330	2980	- 30
J.B.	2425	1000			2810	+385	2580	+155
Avg		1000		+402		+357		+ 87

method employed permits a direct comparison of the ability of various infusions to increase the plasma volume. The data presented indicate that the intravenous injection of pectin solution effectively increases the plasma volume, and that the degree of increase corresponds to the amount injected. Furthermore, the increased plasma volume persists for at least 4 hours after the end of the infusion. In some cases a definite increase in plasma volume was present 24 hours later. These results are in sharp contrast to those obtained following the injection of 1000 cc physiologic saline. Although a larger volume of fluid was injected in the 4 experiments in which physiologic saline was used, the increase in plasma volume at corresponding time intervals was strikingly less than the amount of fluid introduced.

The 5 patients who received pectin and who at the same time received Evans Blue dye (T-1824) for plasma volume determinations all developed a purpuric rash on the ninth day after the pectin was given. In 2 instances the purpura was associated with swelling and moderate pain in several of the large joints. Both joint and skin manifestations disappeared entirely within 5 days. Complete blood and platelet counts, urinalysis and vitamin C blood level determinations were normal in each case. Subsequent reinjection of pectin in the same patients without determining the plasma volume did not produce these symptoms. No such reactions were observed following the saline experiments.

The development of the purpuric skin lesions is probably in some way related to the combination of pectin solution and the dye used in this study, since pectin given alone to the same patients who had previously developed purpura was not associated with this reaction. Pectin solution has been given to 125 patients,⁴ and in no instance have similar changes been observed. The dye given alone was not attended with purpura. The purpuric reactions which we have observed are being investigated further.

The results of this investigation indicate that pectin when injected intravenously is effective in producing marked and sustained rises in the plasma volume of normal individuals, and that pectin deserves intensive study to determine its value as a blood substitute for the treatment of patients in shock.

⁴ Personal communication, F. W. Hartman, M.D., Henry Ford Hospital, Detroit, Mich.

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A Green Pigment-Producing Compound in Urine of Pyridoxine-Deficient Dogs.

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Lepkovsky and Nielsen¹ reported that pyridoxine-deficient rats excrete an unidentified compound which can be converted to a green pigment by ferric ammonium sulphate. Administration of pyridoxine to the deficient rat stops the excretion of the precursor of the green pigment in a matter of hours.

To see if pyridoxine deficient dogs excrete a similar compound these studies were instituted.

Methods. The basic diet consisted of the following: Washed casein 41.4 g, sucrose 29.6 g, Crisco 25.7 g, bone ash 2.0 g, and salt mixture No. 185² 1.3 g. The adult mongrel dogs were allowed to eat as much of the diet as desired. The dogs received daily by dropper: thiamine chloride and riboflavin 60 μ g per kilo; nicotinic acid, 2 mg per kilo; calcium pantothenate, 0.6 g per kilo, and choline, 10 mg per kilo, in addition to vitamins A and D.

Red blood cell counts, hemoglobin determinations (Evelyn), and white blood counts were made at weekly intervals. Hematocrit measurements were made by use of Wintrobe tubes. Daily 24-hour urine specimens were tested for the green pigment-producing substance. To filtered urine made alkaline by addition of ammonium hydroxide, ferric ammonium sulphate was added to bring out the color.

Results. A small amount of green pigment-producing compound could be demonstrated in urine after the 23rd day on a diet in one dog, and after 25 days in the other. The amount was small compared to that in the urine of pyridoxine-deficient rats, but it could be concentrated chromatographically¹ and found to behave similarly to the substance in the rat's urine. The compound did not increase during the period of study, although in one dog the red blood cell count, hemoglobin, and hematocrit dropped from 4.5 million, 10.8 g and 31 cc to 1.83 million, 2.9 g and 9 cc, respectively, by the 107th day of the diet. In the other dog the red blood cell count, hemoglobin,

¹ Lepkovsky, Samuel, and Nielsen, Elmer, *J. Biol. Chem.*, in press.

² McCollum, E. G., and Simmonds, N., *J. Biol. Chem.*, 1918, **33**, 55.

and hematocrit dropped from 6.8 million, 15.3 g and 47 cc to 2.13 million, 4.2 g and 11.5 cc before pyridoxine was administered on the 116th day.

Pyridoxine, 60 μ g daily by mouth, was followed by a rise of reticulocytes to 14.4% and a rapid rise in red blood cells and hemoglobin. After 18 days of therapy the red blood cell count was 4.7 million, hemoglobin 11.5 g, and hematocrit 42 cc. The green pigment-producing compound disappeared from the urine within 48 hours.

Pyridoxine-deficient rats whose urine yields large amounts of green pigment-producing substance do not develop such profound anemia as the dogs. Fifteen pyridoxine-deficient rats had hemoglobin values varying from 11.4 to 14.2 g per 100 cc, averaging 13.0 g. The hemoglobin of similar pyridoxine-fed rats varied from 15.2 to 16.5 g, averaging 15.8 g per 100 cc.

Conclusions. 1. Pyridoxine-deficient dogs excrete small amounts of a compound which can be converted to a green pigment by ferric ammonium sulphate. 2. Pyridoxine-deficient rats do not develop as profound anemia as the deficient dogs.

We wish to thank Mrs. D. Parsons for making the hemoglobin determinations and red cell counts on the rats.

13752 P

Preservation of Avian Malaria Parasites by Low-Temperature Freezing.*

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Since malaria plasmodia cannot be readily cultivated it has been necessary to maintain them in laboratory animals wherever they are used for research or classroom material, and hence Coggeshall's¹ demonstration that malaria parasites of the monkey could be preserved for at least 70 days[†] in the frozen state seemed to have much potential importance. For laboratory use the avian malaras are even more widely employed, and so experiments were undertaken to apply this technic to them.

* The authors wish to acknowledge with gratitude a grant-in-aid from Sigma Xi.

¹ Coggeshall, L. T., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 499.

[†] Later extended to 151 days. Horsfall, F. L., *J. Bact.*, 1940, **40**, 559.

So far *Plasmodium cathemerium*, *circumflexum*, *hexamerium*, *lophurae*, *nucleophilum*, *relictum* var. *matutinum*, *rouxi*, and *vaughani* have been successfully preserved in this way. A total of 32 cases have been infected with parasites frozen for periods up to 90 days. For *lophurae*, ducks served as hosts; in all other cases female canaries were used.

The technic of freezing and thawing was not different than that used by Coggeshall and others, except that a small electric rotator was used to rapidly revolve the tubes containing the infective mixture during the freezing and thawing process. For convenience the freezing was done in a beaker of alcohol maintained at the correct temperature by being kept in the freezing cabinet, rather than in a specially prepared mixture of alcohol and solid carbon dioxide. Thawing was done in water at 42°C. Small Pyrex test tubes measuring 10 x 1 cm were used for containers, and the amount of blood frozen at a time was generally about 300 cmm. It appeared to make little difference whether this was defibrinated, or diluted with a little physiological citrate solution.

The most important factors in maintaining viability seemed to be speed in the freezing and thawing process, but—contrary to the claims made by some others—fairly considerable variations in temperature did not appear to be of great importance. It was found that the temperature of the alcohol in which the tubes were immersed varied occasionally as much as 25°C, depending on whether dry ice had been freshly added or was getting low. Temperature determinations made under these two conditions showed a range of from about -78°C to -55°C.

No differences were observed in the ability of different species of plasmodia to withstand freezing, nor is there reason to think that they cannot be maintained for periods considerably longer than the maximum so far obtained of 90 days. Coggeshall's success in keeping monkey plasmodia for 70 days has already been cited, and recently Kessler² has been able to preserve *Bartonella muris* in this way for 11 weeks.

Nor does the virulence of the plasmodia seem much affected. Incubation periods are in some cases quite long, as, for example, when *Plasmodium nucleophilum* was kept frozen for 60 days. Ordinarily parasites would have been demonstrable within a week or less, but in this case almost two weeks was required. This suggests that a considerable proportion of the parasites are killed. Microscopic examination of frozen material shows many destroyed erythro-

² Kessler, W. R., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 238.

cytes (at least in stained smears) and apparently many injured parasites, but there are others which look quite normal. The periodicity of *Plasmodium relictum* var. *matutinum*, normally very sharp, did not seem to be altered in any way.

Summary. Seven species of avian malaria plasmodia have been successfully preserved for periods up to 90 days by low-temperature freezing. Very rapid freezing and thawing seem of more importance than the occurrence of fairly considerable temperature variations. For maintaining stocks of malaria for research or class use this method of preservation has much potential importance. It is probable that all species of plasmodia can be preserved in this way equally well. And it does not appear that different stages of the parasite are much differently affected.

13753

Apparatus for Rapid, Sterile Removal of Chick Embryos from Eggs.

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Developing chick embryos are now used extensively for the study of viruses and rickettsiae. At present they are used in large numbers for the preparation of vaccines against virus and rickettsial diseases. However, it has always been extremely difficult to sterilize thoroughly the eggshell with ordinary chemical disinfecting agents. In 1939 Penna¹ described a technic in which he employed an oxyacetylene torch for burning the eggshell open while the egg was rotated slowly in an adjustable clamp turned by an electric motor. Penna's technic is satisfactory except that it is too slow. The purpose of this report is to describe a modification of Penna's apparatus which permits a considerably faster removal of embryos under sterile condition.

The apparatus is illustrated in Figs. 1, 2, and 3. The eggs are handled in groups of four carried in shallow metal trays (A). The flame of an oxyacetylene torch (B) is applied at the assumed position of the air sac's margin as each egg is made to rotate through one complete revolution; the optimum rate of turning is dependent on the resistance to burning shown by the shells of a particular lot

¹ Penna, H. A., *Am. J. Trop. Med.*, 1939, **19**, 589.

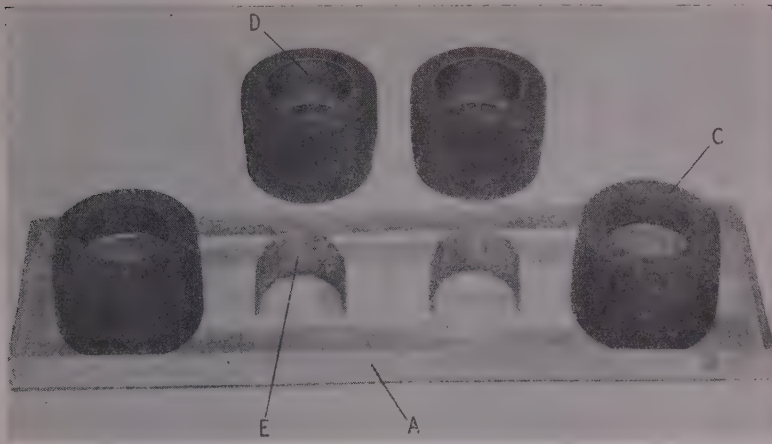


FIG. 1.

Metal tray, showing four guides and the egg holders which successively rotate about them as the holders come into contact with a turning disk. The holders consist of rubber tubing and a short section of thin metal tubing.

of eggs, but is usually of the order of 45 rpm. The metal trays serve to retain the embryonic fluids which occasionally issue forth when a shell cap breaks loose while being flamed. They also perform the same function while the caps are being displaced with a sterile instrument and while the embryos are being removed. The trays and holders can be quickly rinsed in running tap water and then put back into service.

The details of the holders and trays can be seen in Fig. 1. Each holder (C) consists of a $1\frac{5}{8}$ -in. length of heavy rubber tubing into one end of which is inserted a 1-in. length of brass tubing (D). The outside diameters of the rubber and metal are $2\frac{1}{4}$ in. and $1\frac{1}{2}$ in. respectively; the wall thicknesses, $\frac{3}{8}$ and $\frac{1}{32}$ in. respectively. Trays (A) are of 0.025-in. monel metal, 12 in. in length, $3\frac{1}{2}$ in. in width, and $\frac{5}{8}$ in. in height, soldered on the inside at each corner. Equally spaced along each tray are 4 soldered monel metal guides (E) about which the holders can rotate fairly closely but freely. The guides taper in angular width from their tops to their bases, 2 of them being obtained by sawing through a 1-in. length of $1\frac{1}{2}$ -in. tubing at an angle of about 20° to the axis of the tubing. All soldering must be done with a soldering iron rather than a flame to avoid permanently distorting the trays.

Loaded trays are pulled between 2 metal guiding strips (F, Fig. 2), $\frac{1}{4}$ in. in height, across the top surface of a table-like stand (G) which is covered with sheet metal. Each egg holder in succession

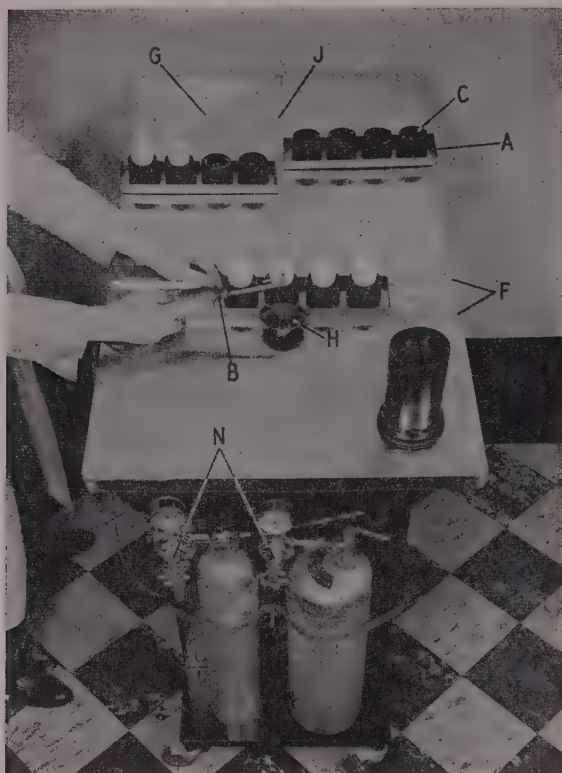


FIG. 2.

Apparatus in operation, showing the oxyacetylene torch being applied to incubated eggs as the first step in the removal of the shell caps and the sterile extraction of the embryos.

comes into contact with a rotating $3\frac{1}{8}$ -in. disk (H) which causes the holder itself to turn about its own guide. Except at its hub, this disk is about $\frac{1}{8}$ in. thick and of slightly conical shape for purposes of drainage. The peripheral edge is rounded and knurled. With ample lateral freedom the shaft passes to a motor (I) beneath the top through a hole of considerably larger diameter, into which is forced a thin protective metal sleeve that extends almost to the disk. A third metal strip (J) serves to separate the incoming and outgoing trays. The left edge of the left guiding strip is provided with a downward slope, and one can with a little practice learn to maneuver the trays quickly with one hand into or out of the guiding channel without disturbing the orientation of the eggs.

The motor (I, Fig. 3) is a universal, 110-volt, variable speed

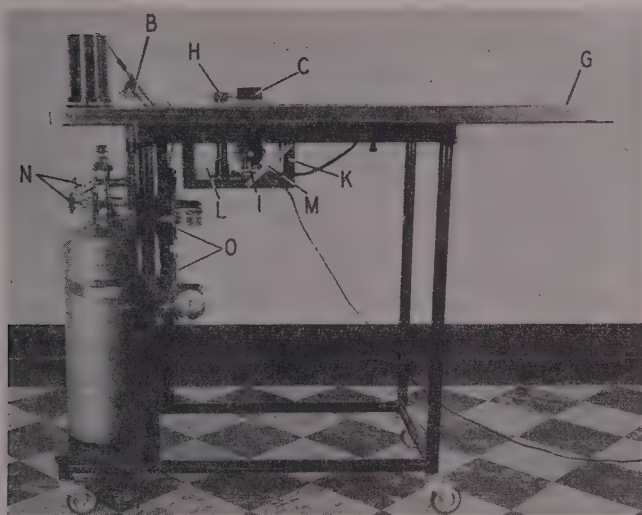


FIG. 3.

Side view of apparatus, showing the system of gas pressure regulation and the method of mounting below the top surface a low-speed motor which furnishes the power for the turning disk that engages the egg holders.

type of 1/30 horsepower, with a built-in gear reduction system yielding a shaft speed of approximately 30 rpm at rated load, or roughly 60 rpm with no applied load. A rheostat regulates the speed. A hinged motor mounting (K) permitting lateral movement, a coil spring (L) for lateral tension, and a limiting stop (M) complete the essential mechanism. However, some form of slip clutch to protect the gears is recommended.

The main stem of the torch is formed from 3/16-in. copper tubing, and the brass burner tip is provided with a jet hole .02 in. in diameter. For the first stage of reduction the oxygen and acetylene regulators (N) are initially adjusted to supply the respective pressures of 20 and 10 lb per sq in. A second stage of reduction is furnished by two low-pressure Hoke Phoenix regulators (O), each with fixed adjustments which insure a steady gas supply at a pressure of 2 lb per sq in. Final regulation is made with needle valves. Adjustments once properly made do not need to be repeated. The regulating system described has given satisfactory service over a long period of time.

Recently Dr. Kenneth Goodner in this laboratory has found that ordinary illuminating gas can be substituted for the acetylene, and in spite of the lower flame temperature it appears to work equally

well with a hole of .028-in. diameter. Furthermore, the gas pressures apparently do not have to be adjusted as critically as with the hotter flame. The illuminating gas is taken directly from the laboratory supply line, and only a single regulator and the needle valve at the torch are used to control the flow of oxygen.

Extensive experience with the apparatus during more than a year of operation has shown that a single operator can take care of from 1000 to 1200 eggs per hour.

13754

Toxicity of 3,3'-Methylenebis (4-hydroxycoumarin).

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Dicumarol,* chemically 3,3'-methylenebis(4-hydroxycoumarin), is the hemorrhagic principle isolated by Link and his coworkers.^{1, 2} It can now be prepared synthetically by improved methods.³ The substance is responsible for the "sweet clover disease" of cattle,^{4, 5} and lowers prothrombin level of blood plasma.⁶⁻¹⁰ In view of the recent clinical interest in dicumarol,¹¹⁻¹⁶ a systematic study of its toxicity became highly desirable.

* Dicumarol is the collective trademark of the Wisconsin Alumni Research Foundation, which controls the use thereof.

¹ Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 21.

² Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 513.

³ Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 529.

⁴ Schofield, F. W., *J. Am. Vet. M. A.*, 1924, **17**, 553.

⁵ Roderick, L. M., *J. Am. Vet. M. A.*, 1929, **27**, 314.

⁶ Roderick, L. M., *Am. J. Physiol.*, 1931, **96**, 413.

⁷ Quick, A. J., *Am. J. Physiol.*, 1937, **118**, 260.

⁸ Campbell, H. A., Roberts, W. L., Smith, W. K., and Link, K. P., *J. Biol. Chem.*, 1940, **136**, 47.

⁹ Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 1.

¹⁰ Overman, B. S., Stahmann, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, 1942, **142**, 941.

¹¹ Butt, H. R., Allen, E. V., and Bollman, J. L., *Proc. Staff Meet., Mayo Clin.*, 1941, **16**, 388.

¹² Bingham, J. B., Meyer, O. O., and Pohle, F. J., *Am. J. Med. Sc.*, 1941, **202**, 563.

¹³ Meyer, O. O., Bingham, J. B., and Pohle, F. J., *J. A. M. A.*, 1942, **118**, 1003.

Single Doses. Mice, rats, and guinea pigs were employed. For both intravenous and oral administration, a fresh stock solution of the disodium salt of Dicumarol was prepared according to Professor Link's directions.¹⁷ All doses were calculated on the basis of Dicumarol.

Animals receiving lethal doses by intravenous injection died in 3-4 hours. Amounts greater than lethal doses caused practically immediate death preceded by a series of clonic convulsions. Most animals surviving sublethal doses showed a prolonged period of respiratory depression. The symptomatology after oral administration was similar to that after intravenous injection, but the period of intoxication was longer. Death usually occurred in 3-4 days, or complete recovery took place. Bleeding from the nose and anus was apparent in many animals. All surviving animals were observed for a week before the experiment was concluded. The median lethal doses ($LD_{50} \pm$ standard error) determined intravenously and orally in mice and rats, and intravenously in guinea pigs, are shown in Table I.

Prolonged Administration. Four groups of 5, 6, 5, and 5 dogs, each received daily doses of 50, 20, 10, and 5 mg per kg, respectively. Their weight varied from 5 to 13.8 kg, average 9.14 kg. Dicumarol dispensed in gelatin capsules was given by mouth. All animals died within 28 days. Careful necropsies were performed, and microscopic examinations of viscera routinely made. Hemorrhage into various tissues and organs (stomach, intestines, lungs, thymus, and serous cavities), and pulmonary edema were present in most of the dogs. Of the 21 animals, 5 presented microscopic evidence of central necrosis of the liver—moderate in 1 but slight in the remaining 4.

Five groups of 5 rabbits each were injected by the marginal ear

TABLE I.
Acute Toxicity of Dicumarol.

Animal	Avg wt, g	Administration	No. animals used	$LD_{50} \pm$ S.E. mg per kg
Mice	16.2	Intravenous	32	64.30 ± 6.11
		Oral	48	232.8 ± 46.56
Rats	84.5	Intravenous	32	52.13 ± 1.79
		Oral	48	541.6 ± 67.7
Guinea Pigs	251.3	Intravenous	32	58.60 ± 2.29

¹⁴ Barker, N. W., Butt, H. R., Allen, E. V., and Bollman, J. L., *J. A. M. A.*, 1942, **118**, 1003.

¹⁵ Townsend, S. R., and Mills, E. S., *Canad. Med. A. J.*, 1942, **46**, 214.

¹⁶ Lehmann, J., *Lancet*, 1942, **1**, 318.

¹⁷ Link, K. P., private communication.

vein with daily doses of 2, 1, 0.5, 0.25, 0.1 mg per kg, respectively. Their body weight ranged from 1.75 to 2.14 kg, average 1.906 kg. The experiment was intended to last 6 weeks. All rabbits on daily doses of 1 and 2 mg per kg died within 10 days except one receiving the smaller dose, which died on the 29th day. Two out of 5 animals on 0.5 mg per kg also died on the 29th day. One out of 5 animals died after the 15th dose of 0.25 mg per kg. The other animals survived the entire experiment, namely 42 days. Necropsies of the rabbits that died revealed a uniform occurrence of hemorrhage and pulmonary edema. Out of 13 animals, 6 showed central necrosis of the liver—4 moderate and 2 slight. All the animals surviving 42 doses of 0.5, 0.25, and 0.1 mg per kg were sacrificed for postmortem examination, and found normal.

Five groups of 5 mice each ingested various concentrations of Dicumarol in food—0.5, 0.1, 0.05, 0.01, and 0.005%, respectively. The majority of them died within 9 days; others within 23 days; and 2 survived 30 days on 0.005% of the drug in food. Of the 23 mice which died from Dicumarol, hemorrhage and pulmonary edema were the prominent features, while central necrosis of the liver occurred in 2 animals. The 2 surviving animals were normal.

Six groups of 5 rats each were similarly fed Dicumarol in food—1, 0.5, 0.1, 0.05, 0.01, and 0.005% respectively. Twenty-one animals died within 9 days; 4 within 18 days; and 1 on the thirty-first day. One rat survived 30 days on 0.01% of Dicumarol, and 3 on 0.005%. Hemorrhage into various tissues and organs, and pulmonary edema were observed in most rats. Of the 25 animals examined which died from the toxic action of Dicumarol, 13 showed central necrosis of the liver. An example is given in Fig. 1. No pathologic changes could be detected in the 4 rats surviving 30 days of medication.

Comment. The above data indicate very clearly that Dicumarol is a drug toxic to animals, particularly by prolonged administration. As in the "sweet clover disease" of cattle, hemorrhage into tissues and organs is the most prominent feature of those animals which die from the administration of this substance. Pulmonary edema is also frequently encountered. The rat's liver appears to be more susceptible to Dicumarol than that of other species of animals, often displaying central necrosis, although the same lesion may occasionally occur in mice, dogs, and rabbits—more so in the last. It must be realized, of course, that these experiments were carried out under the most severe conditions, and that the results recorded in the present work should not be used as arguments against further careful

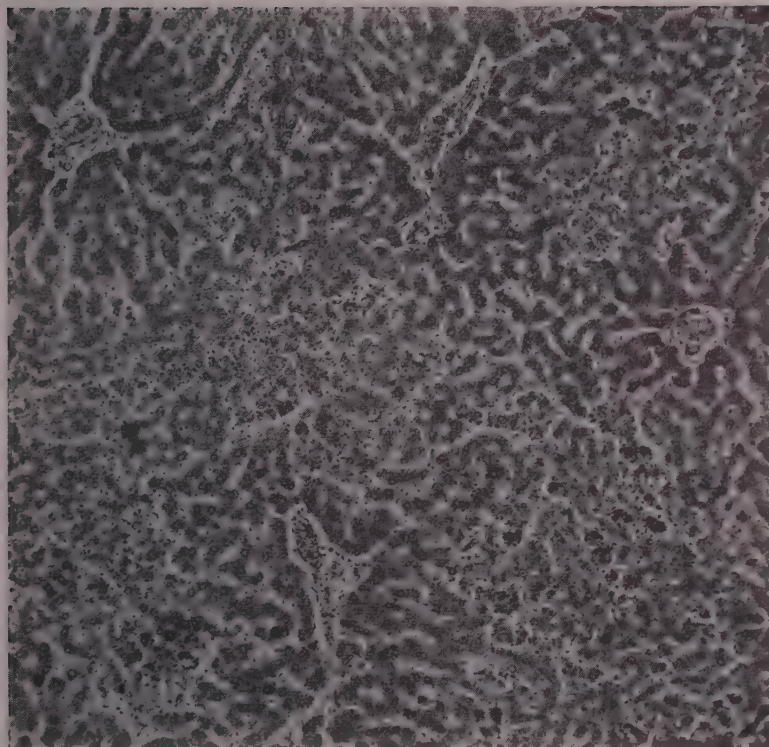


FIG. 1.

Action of Dicumarol on the Rat's Liver.

Rat numbered 16, female, weighing 100 g, was fed Dicumarol, 1% in food. It died on the sixth day. The daily food intake was recorded. The animal had ingested a total of 140 mg of the drug during the first 3 days, and it refused to eat thereafter. At autopsy the liver was found to be most affected. Note the central necrosis and leucocytic infiltration.

clinical trials. There is a possibility that amounts corresponding to therapeutic doses may be harmless. Our plea is the same as that of clinicians;^{18, 19} that is, the administration of Dicumarol must be controlled by definite criteria such as the prothrombin determination and other appropriate tests.

Summary. (1) The median lethal doses of Dicumarol have been determined intravenously or by mouth in mice, rats, and guinea pigs. (2) Death uniformly occurs in rabbits with intravenous injection of daily doses of 1-2 mg per kg; in dogs with oral adminis-

¹⁸ Meyer, O. O., Bingham, J. B., and Axelrod, V. H., *Am. J. Med. Sc.*, in press.

¹⁹ Shapiro, S., Sherwin, B., and Gordimer, H., *Ann. Surg.*, in press.

tration of daily doses of 5-50 mg per kg; and in mice and rats with the feeding of 0.01-1% Dicumarol in food. The majority of rabbits can tolerate daily doses of 0.1-0.5 mg per kg by vein for 6 weeks, and a few mice and rats can survive 30 days on a diet containing 0.005% Dicumarol. (3) Most animals dying from Dicumarol develop hemorrhage into various tissues and organs, and pulmonary edema. Central necrosis of the liver has been observed in about one-half of the rats examined, and occasionally in rabbits, mice, and dogs.

13755

Anesthetic Activity of Some New Derivatives of Barbituric Acid.*

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P. D. Lamson.)

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Nashville, Tenn.*

This is a study of 7 barbituric acids which, so far as we are aware, have not previously been referred to in the literature.

We have found all of these compounds to be anesthetic in mice. The anesthetic and lethal doses are shown in Table I. The corresponding data for two familiar drugs, pentobarbital and evipal, are included for comparison.

Methods. Animals. Male white mice were used in all of the experiments.

Administration of Drugs. All drugs were given as freshly prepared solutions of the sodium salts. (The doses shown in Table I are expressed in terms of the acid form.) The concentrations of the solutions were such that the mice received about 0.012 cc of solution per gram of body weight for the intravenous doses, about 0.017 cc per gram for the intraperitoneal anesthetic doses, and about 0.025 cc per gram for the intraperitoneal lethal doses. In the determination of the intravenous anesthetic doses, the injection occupied a period of $\frac{1}{2}$ minute.

* This work was aided by a grant from Mallinckrodt Chemical Works. The first five compounds listed in Table I were kindly made for us by Mallinckrodt Chemical Works. The other two new compounds were synthesized in this laboratory.

TABLE I.
Anesthetic Doses, Lethal Doses, and Recovery Times as Determined in Mice by
Injection of Solutions of Sodium Salts.

Barbituric acid	Melting point °C, corr.	Intravenous	Intraperitoneal		Recovery† time, min
		AD 50* mg/kg	AD 50 mg/kg	LD 50† mg/kg	
5-isopropyl-5-isobutyl	160-161	63.5 ± 1.5	76.0 ± 1.8	231 ± 4	25
5- <i>n</i> -butyl-5- <i>sec</i> -butyl	135-138	46.6 ± 0.9	63.8 ± 1.2	204 ± 9	16
5- <i>n</i> -butyl-5-isoamyl	170-172	23.7 ± 1.2	116.4 ± 4.8	600 ± 35	1
5- <i>sec</i> -butyl-5-isoamyl	132-133	32.5 ± 0.7	83.6 ± 2.7	243 ± 8	2
5- <i>n</i> -propyl-5- <i>n</i> -amyl	124-128	40.8 ± 0.5	88.6 ± 2.7	232 ± 10	4
5- <i>n</i> -propyl-5- <i>n</i> -hexyl	114-116	48.4 ± 2.6	111.0 ± 2.6	302 ± 13	1
5- <i>n</i> -propyl-5- <i>n</i> -heptyl	112-114	92.4 ± 3.1	132.0 ± 5.7	269 ± 16	15
pentobarbital		33.3 ± 1.9	42.3 ± 0.7	121 ± 2	28
evipal		29.2 ± 1.0	62.5 ± 1.1	286 ± 4	1

* AD 50—Median anesthetic dose.

† LD 50—Median lethal dose.

‡ See *Methods*.

Median Doses. The median anesthetic doses (AD 50) and median lethal doses (LD 50) were estimated by interpolation on the assumption that the curve relating log dose and proportion anesthetized or killed is the integrated normal frequency curve. In Table I the median doses are accompanied by their standard errors, calculated by the equations given by Bliss.¹

Criterion of Anesthesia. A mouse was considered anesthetized if it could not gain and maintain the standing position after stimulation by repeated pinching of the tail.

Recovery Times. A dose 1.25 times the previously determined AD 50 was given intravenously. The interval between the end of the injection and the recovery of the degree of righting ability described above was measured. The value shown in Table I is the median recovery time observed in a series of 11 mice.

The anesthesia produced in mice by all of the new compounds except isopropyl-isobutyl-barbituric acid is as quiet as that produced in this animal by any of the well known barbituric acids. Except for a fine rapid tremor of the legs which is sometimes seen in the light stages of anesthesia, the mice are well relaxed. The anesthesia with isopropyl-isobutyl-barbituric acid is not so smooth. Clonic spasms of the legs are much more pronounced, and occasionally a brief generalized convulsion can be elicited by stimulation. These convulsive movements are more conspicuous with small doses, large doses producing a rather quiet anesthesia. In mice the convulsive effects of this drug are no more intense than those of some of the widely used barbituric acids (*e. g.*, dial and evipal).

¹ Bliss, C. I., *Quart. J. Pharm. and Pharmacol.*, 1938, **11**, 192.

The recovery times shown in Table I, which furnish a rough index of the relative persistence of action of the compounds, indicate that they are all rather short acting. The longest acting of the new compounds has a duration of action of the same order as that of pentobarbital; the shortest acting, of the same order as that of evipal.

The first 3 of the new compounds show a brief delay in the full development of anesthesia after an intravenous injection. In the mice receiving the dose 1.25 times the AD 50, the mean lag for these three compounds was, respectively, 3 min, $\frac{1}{2}$ min, and 5 sec. The other 4 new compounds had no appreciable lag.

Early work with the barbituric acids led to certain inferences regarding the relation between structure and activity. It is these generalizations, doubtless, that have discouraged further investigation of derivatives of the type presented here. For example, the generalizations arrived at by one worker in this field² included these: (1) That the sum of the carbon atoms in the 2 substituent groups in the 5-position is 7 in the most effective compounds; (2) that the greatest dissimilarity in the 2 groups gives the greatest activity; (3) that if both groups are larger than ethyl, less effective compounds result. The activity of the compounds studied here shows that none of these rules is generally applicable.

Summary. The anesthetic activity in mice of seven new 5,5-dialkyl-barbituric acids has been studied. Intravenous anesthetic doses, intraperitoneal anesthetic doses, intraperitoneal lethal doses, and duration of action were measured.

13756

Nature of the X-ray Effect in CO Recovery.*

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Rapid recovery from severe carbon monoxide poisoning under X-ray treatment^{1, 2} and spectrographic evidence of the reduction of

² Shonle, H. A., *Ind. Eng. Chem.*, 1931, **23**, 1104.

* This study was aided by a grant from the Research Council of the University of Missouri.

¹ Cameron, John A., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 29.

² Cameron, John A., *Radiology*, 1941, **36**, 486.

blood CO after X-ray treatment³ have previously been reported. A recent publication has described progressive discontinuous stages of decreased resistance to CO among young rats and rabbits during the first few days after birth.⁴

Some attempts have lately been made to determine the factors responsible for these phenomena. Each of several kinds of effects has been considered and there may be others not yet investigated.

The release of reserve red cells from the spleen in response to X-ray injury to circulating cells has often been described,⁵ but a latent period, or lag, of 6 hours or more is associated with this process. In CO recovery the effect must be maximal within so short a time after the onset of asphyxia that the release of this reserve seems to be ruled out.

Possible recombination of CO and O₂ in the blood and tissues to form CO₂ and reduce the amount of CO present was checked in 12 experiments carried on in CO and O₂ saturated water at body temperature. In none of these tests was the amount of CO₂ (determined as carbonate) significantly increased by X-ray exposures of 500-2,000 *r*. Actual differences in cc of standardized HCl used were: 0.4-0.55; 0.45-0.5; 0.07-0.27; 0.35-0.3; 0.45-0.42; 0.4-0.25; 0.18-0.1; 0.1-0.15; 0.2-0.25; 0.1-0.05; 0.2-0.15; 0.3-0.41, an average of 0.09 when a difference of 0.2 might be significant. Dr. Daniel Mazia and Dr. Lloyd B. Thomas kindly advised me in this work. Dr. L. J. Stadler, of the U. S. D. A. and the Department of Field Crops, University of Missouri, generously provided the X-ray services for the work discussed here.

A series of experiments with whole blood of Rhesus monkeys, saturated with CO by shaking in a flask while connected to a tank of CO were next undertaken. Half of each CO saturated sample was placed in each of 2 petri dishes and 1 was exposed to 650-1000 *r*. Spectrographic analysis by Dr. Victor Ells showed no significant difference in the CO content of X-rayed and control samples. Actual readings were 1.17-1.21; 0.90-0.96; 1.11-1.117; 1.47-1.48; 1.17-1.21; and 1.31-1.40; an average difference of 0.05 when a difference of 0.10 is necessary for significance.

The reduction of CO in the blood of intact animals is almost always accompanied by striking changes in the depth and frequency of breathing. The inspirations of animals in deep CO coma are not only much shallower but much less frequent than in similarly

³ Cameron, John A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 558.

⁴ Cameron, John A., *J. Cell. and Comp. Physiol.*, 1941, **18**, 379.

⁵ Downey, Hal, *Handbook of Hematology*, 4 Vol., New York, 1938.

exposed animals after X-ray. The spikes on the kymograph records of "CO plus X-ray animals" are 3 to 5 times as high as those on the records of "CO animals." In monkeys² the rate of inspiration per minute was 78 for "CO animals" and 132 for "CO plus X-ray animals." (Average of data from 6 pairs of experimental animals). These observations suggest that the X-ray effect is brought about by stimulation of the nervous system to produce greater and more effective ventilation at the critical time during recovery.

In newborn rats and rabbits the survival time in CO is 10 times that of the adults, or even more. During the first weeks of life the survival times decrease by 5 discontinuous steps to the adult level.⁴ These shifts in susceptibility take place during the early postnatal period when the development of the nervous system is being completed. The longer survival in CO of younger subjects may be due to the greater resistance of the respiratory center in control during early life. The abrupt changes to shorter survival periods can perhaps be correlated with the shifting of respiratory control to higher and more delicate centers with increase in age.

When revival by X-ray treatment is attempted on rats and rabbits under 8 days of age no favorable results are obtained. In 20 littermate pairs of rats and 19 littermate pairs of rabbits between 1 and 8 days of age no significant prolongation of life among the X-rayed animals was noted. This seems to support the concept of respiratory center stimulation rather than that of direct effect on the respiratory chemistry of the organism. Apparently CO inhibition of the center of respiratory control in very young animals is not subject to X-ray reversal.

It is suggested that during the process of CO poisoning in older animals the respiratory centers may be successively inhibited from higher to lower, reversing the sequence by which they assume control during the first 3 weeks of life.⁴ Inhibition of the usual center of respiratory control in older animals by CO may then be subject to X-ray relief because the X-ray excites to temporary functional activity centers early discarded by the developing animal. These centers need only remain active until the usual adult centers have recovered sufficiently to resume their normal rôle.

13757

Studies on the Anticoagulant
3,3'-Methylene-Bis-(4-Hydroxycoumarin).

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Recent reports on experimental and clinical studies of 3,3'-methylene-bis-(4-hydroxycoumarin), (Dicumarol*), a substance originally isolated from spoiled sweet clover and capable of decreasing the prothrombin content of blood *in vivo*, have indicated the possible application of this material in clinical medicine.

The history, chemical isolation, synthesis and assay of 3,3'-methylene-bis-(4-hydroxycoumarin) have been described in a number of papers by Link and associates.¹⁻⁵ Experimental and clinical investigations have been conducted by Butt and associates,⁶ as well as by Meyer and coworkers.^{7, 8} Further data have appeared in the American⁹ and foreign^{10, 11} literature.

Unlike heparin, Dicumarol does not inhibit blood coagulation *in vitro*. It exerts its anticoagulant effect following oral or parenteral administration, but there is a considerable latent period whichever route is used. This publication deals with its efficacy in preventing thrombosis and its toxicity.

Meyer and coworkers⁷ attempted to demonstrate the ability of the Dicumarol to inhibit experimental thrombosis, but they found

* Dicumarol is the collective trademark of the Wisconsin Alumni Research Foundation, which controls the use thereof.

¹ Campbell, H. A., Roberts, W. L., Smith, Wm. K., and Link, K. P., *J. Biol. Chem.*, 1940, **136**, 47.

² Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, 1941 **138**, 21.

³ Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 513.

⁴ Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 1.

⁵ Overman, R. S., Stahmann, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, 1942, **142**, 941.

⁶ Butt, H. R., Allen, E. V., and Bollman, J. L., *Proc. Staff Meet. Mayo Clin.* 1941, **16**, 388.

⁷ Bingham, J. B., Meyer, O. O., and Pohle, F. J., *Am. J. Med. Sci.*, 1941, **202**, 563.

⁸ Meyer, O. O., Bingham, J. B., and Axelrod, V. H., *Am. J. Med. Sci.*, in press.

⁹ Centr. Soc. f. Clin. Res., *J. A. M. A.*, 1942, **118**, 1003.

¹⁰ Lehmann, J., *Lancet*, 1942, **1**, 318.

¹¹ Townsend, S. R., and Mills, Edw. S., *Canad. Med. Assn. J.*, 1942, **46**, 214.

their methods inadequate. In our experiments we used a method similar to the one developed in Best's laboratory.¹² Thrombosis was produced in the peripheral veins of dogs and the prevention of thrombus formation was attempted in other dogs by premedication of the animals with Dicumarol.

Segments of about one inch in length of the radial and saphenous veins of dogs were injected through the skin with 0.15 or 0.25 cc of ethanolamine oleate (Monolate, Abbott), while they were temporarily excluded from the circulation by finger pressure applied on the proximal and distal ends of the segment. This compression was sustained for 3 minutes and then relieved, permitting restoration of circulation. Two to 4 veins were used in each of 4 animals. These segments of the veins were removed 3 to 7 days after the injection and were examined histologically. The data are assembled in the table. As the results indicate, thrombus formation follows this procedure in almost all cases, particularly when the veins were removed 6 or 7 days after injection.

In the next series of experiments, 4 dogs weighing 6 to 9 kg were given 10 or 25 mg daily of Dicumarol by mouth. Their plasma prothrombin time was determined before and during the administration by Link's⁴ modification of the method described by Quick. We used the whole plasma and a 25 and 12.5% dilution, as recommended by Link and coworkers. Coagulation time of whole blood was measured by the method of Lee and White. (See table.) The prothrombin time of these dogs rose within 36-48 hours and then increased rapidly, while the coagulation time increased less markedly. Three to 5 days after the feeding with Dicumarol was started, Monolate was injected in 3 veins of each dog as described above. The administration of Dicumarol was continued at the same level. The veins were removed 3 to 6 days after the injection through a small incision which was carefully closed, in some cases with the local application of thrombin powder. In spite of these precautions the wounds oozed considerably and a blood transfusion of 100 cc given to 2 of the dogs was of temporary benefit only. All 4 died of anemia due to hemorrhage a few days after the veins were removed. Careful histological examination of all veins was made. As can be seen in the table, 8 of the 12 veins showed no change at all except a hypertrophy of the wall in one of them. A slight fibrin deposit was present in one vein and beginning or moderate thrombus formation in two. Only one vein showed an extensive thrombosis.

¹² Murray, D. W. G., Jaques, M. A., Perrett, T. S., and Best, C. H., *Surgery*, 1937, **2**, 163.

TABLE I.
Thrombus Formation by Intravenous Monolate Injection and Inhibition of Thrombosis by Dicumarol.

Dog	Daily dose, mg	No. of doses	Monolate, cc	Vein removed, days after inj.	Histological findings (thrombosis)	Prothrombin time of 12.5% plasma. Below in () blood coagulation time	
						Normal	On day of monolate inj.
A	0		.25	3	++		
	0		.25	7	+++		
B	0		.15	3	+		
	0		.15	6	++		
	0		.25	3	+		
	0		.25	6	+++		
C	0		.15	3	(+)		
	0		.15	7	+++		
	0		.25	3	(+)		
	0		.25	7	+++		
D	0		.15	3	(+)		
	0		.15	7	+++		
	0		.25	3	+		
	0		.25	7	+++		
I	10	7	.25	3	Negative	40.5"	491.0"
	10	7	.25	5	"	(5'12")	(8'0")
	10	7	.15	5	Hypertrophy of wall; no thrombus.		
II	10	15	.25	6	Negative	33.4"	279"
	10	15	.25	6	+++	(4'20")	(6'30")
	10	15 2x	.25	6	Negative		
III	25	7	.25	3	"	40.9"	196.2"
	25	7	.25	5	Slight fibrin deposit	(5'0")	(7'30")
	25	7	.15	5	Negative		
IV	25	9	.25	6	Negative	32.6"	171"
	25	9	.25	6	+	(5'0")	(5'0")
	25	9	.25	6	(+)		

(+) Some fibrin deposits.

+ Intima damage and beginning thrombosis.

++ Wall adherent thrombus; lumen not completely occluded.

+++ Completely or almost completely occluding thrombus with advanced organization.

Thus, the administration of Dicumarol in the doses selected was capable of greatly reducing the incidence of thrombus formation as compared with untreated controls.

Histological examinations of the tissues of the 4 dogs dying as a result of operation during the Dicumarol administration were performed. The general picture was that of advanced anemia, and of particular interest was the occurrence of fatty infiltration of the liver and scattered zones of necrosis in this organ in 2 of these 4 dogs. While the mechanism of the prothrombin depression pro-

duced by Dicumarol is not yet understood, it is possible that the depression is a result of a direct toxic action on the liver. For this reason a study of liver pathology and liver function appeared indicated. Two dogs were given 10 or 25 mg respectively of Dicumarol for 10 days. The animal having received the 25 mg dose died on the 11th day of internal hemorrhage. Its liver showed a marked cloudy swelling, edema, and areas of beginning necrosis. The other dog was killed and autopsied. Here the liver showed a moderate degree of cloudy swelling, a vacuolization of the cells, but no signs of necrosis. Incidentally, a moderate decrease of hemoglobin and RBC count occurred in most of the animals, even when no signs of bleeding could be detected. The WBC and the urine examination did not show peculiarities.

In order to investigate the possibility of functional liver damage, bromsulfalein retention and intravenous glucose tolerance were studied in 2 dogs weighing 17 and 18 kg respectively. Each dog was injected intravenously twice in a 3-day interval with 900 mg/kg of glucose and the blood sugar determined before and 15, 30, 60, and 120 minutes after the glucose injection. Then, each of the 2 dogs was injected intramuscularly with 30 mg of Dicumarol daily for 6 days. Experiments on dogs had shown that a 2% suspension of the Dicumarol in tragacanth can be injected intramuscularly and is effective. However, it does not seem that this mode of administration greatly hastens the onset of the effect. Oral administration was resorted to for another 9 days on account of pain and swelling following the repeated injections. Prothrombin times were routinely determined. On the last day of the administrations, the glucose tolerance test was repeated but failed to show a significant deviation from the previous control tests in both dogs. Similarly, intravenous bromsulfalein injections with 5 mg/kg given before and after the administration of Dicumarol did not reveal an increased dye retention. The absence of functional liver damage in these experiments is in agreement with clinical findings published by Meyer and associates.⁷

Experiments in Monkeys (Macacus rhesus). One monkey of 4 kg weight was given 5 mg/kg of Dicumarol orally daily and another one of 3.9 kg weight, 10 mg/kg daily. Their plasma prothrombin time rose rapidly from a normal value of 50 seconds for the undiluted plasma to 600-700 seconds within 3 days. While being handled, both monkeys contracted minor external injuries and subcutaneous bruises which resulted in chronic hemorrhage. The animals finally became anemic and had to be killed 8 to 10 days after

the beginning of the experiments. The autopsies showed a picture of marked internal hemorrhage and advanced anemia. One of them showed an increased fat content of the liver and both had a few areas of necrosis in this organ. The other organs were negative except for a marked capillary dilation and a moderate degree of cloudy swelling.

Experiments in Guinea Pigs. Three groups of 6 animals each were formed. The first group was kept on a normal diet, the second one received a daily injection of 50 mg of vitamin C in addition to this diet, and the third group consisted of animals which were kept on a vitamin C-free diet for the last 2 weeks and through the duration of this experiment. All animals were given orally 25 mg of Dicumarol every second day until death occurred. The average survival times for the 3 groups were 5, 4.5, and 3.5 days respectively. While the small number of animals makes a statistical evaluation impractical, a tendency of the vitamin C-depleted animals to succumb earlier to the Dicumarol is apparent. This is in agreement with observations made by Sullivan in Link's laboratory.¹³

Histological examination of the livers in several animals of all 3 groups showed a tendency to fatty infiltration and degeneration and a few foci of early necrosis. An identical picture was observed in guinea pigs kept on a vitamin C-free diet without administration of Dicumarol. Such changes were found to be entirely reversible after normal dietary conditions were restored to the vitamin C-depleted animals. A connection between vitamin C level and sensitivity to Dicumarol must be considered possible.

Comments and Summary. 3,3'-methylene-bis-(4-hydroxycoumarin), Dicumarol, was shown in our experiments to be able to greatly reduce the incidence and degree of thrombus formation following the intravenous injection of Monolate in dogs. In dogs and monkeys some signs of necrosis of the liver were found in a number of animals, but since most of them suffered from a severe anemia, the significance of this finding is questionable. The livers of guinea pigs treated with the sweet clover factor showed changes as they occur in vitamin C-depleted animals. Intravenous glucose tolerance and bromsulfalein retention tests failed to show liver damage in animals not suffering from bleeding due to the administration of the drug. While this is in agreement with clinical findings, further studies of the physiology of this substance with particular reference to liver function appear indicated.

Our present experience shows that there is a considerable difference

¹³ Sullivan, W. R., Doctor Dissertation, Univ. Wisc., Madison, May, 1942.

in tolerance between various species of animals and also among the individuals of the same species. The observation that single blood transfusions in dogs in a state of extreme depression of the prothrombin level were of only temporary benefit, is paralleled by similar clinical findings^{11, 12} and calls for particular care in avoiding excessively high dosage.

13758

Comparative Studies of the Effect of Thiamine Deficiency in
Diabetic and Non-Diabetic Rats.

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(Introduced by Elliott P. Joslin.)

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Certain workers^{1, 2, 3} have claimed amelioration of human diabetes with the use of thiamine, particularly when given in combination with other vitamins. Martin⁴ reported intensification of diabetes in depancreatized dogs on a vitamin B-free diet, with remarkable improvement in carbohydrate tolerance when thiamine and flavin were given. On the other hand, in normal rats, Lepkovsky, Wood and Evans⁵ found the glucose tolerance impaired only in the later stages of deficiency and McIntyre and Burke⁶ noted very little change in glucose tolerance, either with deficiency or excess of vitamin B.

The present study was undertaken to determine the effect of deprivation of thiamine on the diabetic condition of depancreatized rats and to ascertain whether or not diabetic animals, when deprived of thiamine, develop signs of deficiency more readily than those without diabetes. It was hoped that the results obtained might elucidate the possible rôle of vitamin B, and specifically of thiamine, in the peripheral neuritis encountered in patients with severe, uncontrolled diabetes.⁷

¹ Vorhaus, M. G., Williams, R. R., and Waterman, R. E., *Am. J. Digest. Dis. and Nutrition*, 1935, **2**, 541.

² Labbe, M., Nepveux, F., and Gringoire, J. D., *Bull. Acad. de Med.*, 1933, **109**, 689.

³ Mosonye, J., and Aszodi, Z., *Klin. Wchnschr.*, 1938, **17**, 337.

⁴ Martin, R. W., *Z. Physiol. Chem.*, 1937, **248**, 242.

⁵ Lepkovsky, S., Wood, C., and Evans, H. M., *J. Biol. Chem.*, 1930, **87**, 239.

⁶ McIntyre, A. R., and Burke, J. C., *J. Pharm. and Exp. Therap.*, 1938, **64**, 465.

⁷ Joslin, E. P., Root, H. F., White, P., and Marble, A., *Treatment of Diabetes Mellitus*, 7th Edition, Philadelphia, Lea & Febiger, 1940, 254-257.

This neuropathy clinically seems comparable to the peripheral neuritis in known vitamin B deficiency associated with beriberi, alcoholism, pregnancy and gastro-intestinal disturbances. In this connection, Meiklejohn,^{8,9} citing the findings of certain recent investigators,^{10,11} concludes that polyneuritis results not from a deficiency of thiamine, but from a lack of other factors present in whole yeast. He states that the only certain consequence of pure thiamine lack is a disturbance of carbohydrate metabolism, resulting in impaired glucose tolerance and the accumulation of lactic and pyruvic acids in the tissue.

Methods. Male white rats of the Yale strain, from 3 to 10 months old, were used. All had been depancreatized at the age of 27 to 37 days.¹² Diabetes was proven in all rats by glucose tolerance tests, although not all animals excreted sugar on the ordinary dog chow diet. The rats were placed in individual metabolism cages. Food, water, urine and urinary sugar were measured daily and the animals were weighed twice weekly.

Prior to the metabolism study, all diabetic animals were maintained on a stock dog chow* diet, with a daily supplement of 5 g of raw beef pancreas. At the start of the experiment they were placed on a vitamin B-free ration consisting of sucrose 54%, casein (Labco, vitamin-free) 35%, Crisco (Primex) 5%, cod liver oil 2%, and Phillips & Hart salt mixture No. 1¹³ 4%. The available carbohydrate of this diet is 75%.

Glucose tolerance tests were done under narcosis with sodium pentobarbital after a fast of 17 hours. Blood from the tail for determination of sugar content was taken fasting and at intervals of ½, 1, 2, 3, and 5 hours after the intraperitoneal injection of 0.35 g of glucose per 100 g body weight in the form of 10% solution. The sugar estimations were done by the Folin-Malmros method.¹⁴

Fourteen diabetic and 5 non-diabetic rats were maintained for 3 to 4 weeks on the diet outlined above together with 25 γ of thiamine,

⁸ Meiklejohn, A. P., *New Eng. J. Med.*, 1940, **223**, 265.

⁹ Meiklejohn, A. P., *New Eng. J. Med.*, 1941, **224**, 420.

¹⁰ Williams, R. D., Mason, H. L., Wilder, R. M., and Smith, B. F., *Arch. Int. Med.*, 1940, **60**, 785.

¹¹ Elsom, K. O'S., Lukens, F. D. W., Montgomery, E. H., and Jonas, L., *J. Clin. Invest.*, 1940, **19**, 153.

¹² Shapiro, R., and Pineus, G., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 416.

* Pratt's, stated by the manufacturers to contain crude protein 22%, crude fat 3%, crude fibre 5%, carbohydrate 61% and nitrogen-free extract 56%.

¹³ Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

¹⁴ Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115.

20 γ of riboflavin, 200 mg of autoclaved rice polish concentrate and 50 mg of choline chloride daily. At the end of this period control glucose tolerance tests were carried out. Following this, the rats were deprived of thiamine while continuing to receive the other supplements daily as before.

Results. Anorexia, lassitude and weight loss appeared at about 10 days after deprivation of thiamine. The acute stage of deficiency ("polyn neuritis") characterized chiefly by paralysis of the hind legs, inability to walk and (if untreated) death within several hours, was reached on the average at about the 40th day of thiamine deprivation. With the diabetic animals this averaged 39 days and with the non-diabetic rats, 45 days. If, however, one non-diabetic rat which carried on for 65 days be excluded, the average length of time for this group was 41 days. Furthermore, that the diabetic animals were not in as good general condition as the non-diabetic rats at the start of the experiment is shown by the fact that the average weight of the diabetic rats was 301 g as compared with that of 405 g for the non-diabetics, the ages of which were comparable. No significant correlation between the severity of diabetes and the time of development of signs of deficiency was apparent.

At the beginning of the period of deprivation, 9 of the 14 diabetic rats were excreting sugar in the urine. As anorexia developed and the food intake became less and less, glycosuria gradually diminished. Glucose tolerance tests done just before the period of deprivation and at approximately 10-day intervals until the development of polyn neuritis, showed no consistent change. The tests performed on the 14 diabetic rats after approximately 3 weeks of deprivation showed an impairment of tolerance in 5 animals, improvement in 3 and no significant change in 6. No significant change in tolerance was noted among the 5 non-diabetic rats studied after 3 weeks of deprivation. Two diabetic and two non-diabetic rats, with which it was possible to carry out glucose tolerance tests at the time of acute polyn neuritis, showed impairment.

When signs of marked deficiency had definitely developed, each animal not sacrificed was given 100 γ of thiamine orally or subcutaneously daily for 10 days. Improvement began in 4 to 8 hours and recovery from weakness and anorexia took place within 2 or 3 days of therapy. The animals gained weight rapidly. After 10 days the thiamine allowance was reduced to the original amount of 25 γ daily for 2 or 3 weeks. Then to 5 of the animals, thiamine in excess (200 γ daily) was given for 10 days. Glucose tolerance tests carried out before and after the administration of 200 γ of thiamine

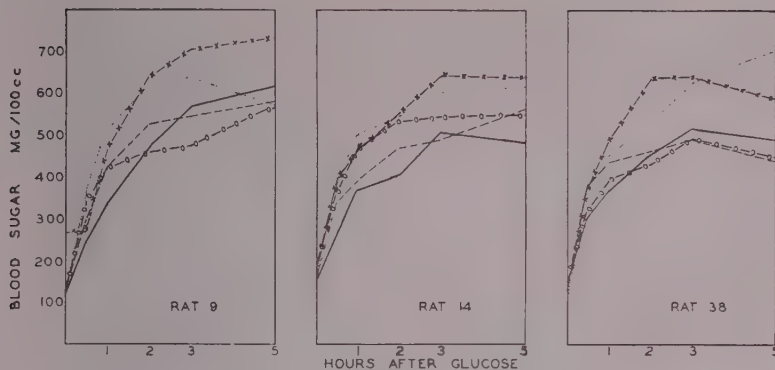


FIG. 1.

Improved Glucose Tolerance in 3 of 5 Diabetic Rats Given Excess Thiamine Following Period of Deprivation.

- Control glucose tolerance curve.
- G.T. curve after 23 days deprivation of thiamine.
- o—o—o G.T. curve during recovery period, after 6 days 100 γ thiamine daily.
- x—x—x G.T. curve after return to maintenance dose of 25 γ thiamine daily for 2-3 weeks.
- G.T. curve after 10 days resumption of excess thiamine, 200 γ daily.

daily showed a significant improvement in tolerance in 3 of the animals when compared with that following the period of 25 γ daily. (See Fig. 1.)

Conclusions. 1. No significant difference was observed in the length of time required by diabetic and non-diabetic rats on a thiamine-free diet to develop signs of marked deficiency (39 as compared with 41 to 45 days respectively). Following treatment with thiamine, the diabetic rats recovered as quickly as did the non-diabetic animals. 2. During the period of deprivation, the glycosuria of the diabetic rats diminished, presumably because of the lowered food intake. However, urinary sugar tended to reappear at the final stage of deficiency at the time of "polyneuritis". Carbohydrate tolerance in both the diabetic and non-diabetic rats, as judged by glucose tolerance tests, showed little change until signs of marked deficiency appeared, when there was impairment of tolerance. 3. Following deprivation, a thiamine intake 4 to 8 times the maintenance dosage appeared to improve carbohydrate tolerance.

Intravenous and Subcutaneous Administration of Alkali-Treated Bovine Serum Albumin to Man and Lower Animals.

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In previous papers we have recorded the results of the administration of bovine serum albumin to human beings¹ and to lower animals.² The present report is concerned with the use of alkali-treated bovine serum albumin in man and other animals.

Materials. Bovine serum albumin prepared by the method already described¹ was treated with sodium hydroxide solutions. The details of the method will be described in a later communication. The sterility of the solutions was ascertained before they were used for injection. The albumin content of the untreated serum was 4 g % and of the alkali-treated serum 3 g %.

Antigenicity. Male guinea pigs were given 3 subcutaneous injections of alkali-treated albumin at suitable intervals. Three weeks later, a shocking dose of 1 cc was given by the intracardiac route. None of the animals died. Only very mild symptoms of anaphylaxis such as excitement and rubbing of the nose were noted. Control guinea pigs sensitized to untreated albumin revealed typical evidences of anaphylactic shock when the final intracardiac injection was given. Several animals died. The surviving animals displayed severe spasms of the abdominal muscles and dyspnea and were noticeably sick and listless for several days. Male white mice (hybrid strains) were sensitized by subcutaneous injections to untreated albumin. When the final injection was given through the dorsal tail vein, typical anaphylactic phenomena occurred. A parallel series of male white mice (hybrid strains) which received subcutaneous injections of treated albumin displayed few or no evidences of anaphylaxis when the final intravenous injection was made. Two dogs which had received treated albumin by vein 2 weeks previously, when given a second intravenous injection without anesthesia exhibited only mild transitory excitement and no typical anaphylaxis.

Toxicity. Twelve white mice given intraperitoneal injections of

¹ Davis, H. A., Eaton, A. G., and Williamson, J., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 96.

² Davis, H. A., and Eaton, A. G., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 20.

treated albumin in relatively large amounts (1 cc per 30 g of body weight) and 6 rabbits given intravenous injections (1 cc) showed no toxic symptoms.

Effectiveness for Blood Replacement. To determine the effectiveness of alkali-treated albumin as a blood substitute, 3 dogs weighing from 3.2 kg to 4.8 kg were used. Under ether anesthesia the right carotid artery was cannulated to record the blood pressure. Amounts of blood were removed equivalent to one-third to one-half of the calculated blood volume of each animal. When the lowered blood pressure showed no tendency to rise, alkali-treated albumin in solution was administered by vein in amounts sufficient to replace the blood which had been removed. The blood pressures rose almost to the initial level, where they were maintained. All of the animals recovered without any untoward effects and are alive at the time of writing.

Administration to Human Beings. Alkali-treated serum albumin which had been subjected to preliminary tests for sterility and toxicity was administered to human beings. In 67 male and female subjects, skin tests for sensitivity were carried out by the subcutaneous and intracutaneous injection of 0.5 cc of the albumin solution. Control injections of 0.5 cc of sterile distilled water were given. The skin tests were negative in all cases. Three men (H.C., J.J., and H.S.) were each given 50 cc of alkali-treated bovine serum albumin by vein at a rate of 5 cc per minute. In 2 of the individuals the blood pressure (systolic and diastolic) rose 6 to 14 mm Hg above the initial level during the injection and slowly returned to this level after the completion of the injection. In the third subject the blood pressure (systolic and diastolic) did not change during or after the injection. The pulse rates were not significantly affected by the injections. Urine collected from each subject after administration of the alkali-treated albumin showed no albuminuria. One individual (H.S.) had received an intravenous injection of untreated bovine serum albumin (100 cc) 3 months previously. In this subject, as in the others, no evidences of anaphylaxis or toxicity could be observed. Preliminary cross-matching tests were not performed.

Summary and Conclusion. Alkali-treated bovine serum albumin when administered by vein or by other parenteral routes is not toxic to human beings, dogs, rabbits, guinea pigs, or mice. It possesses little or no antigenicity. It is capable of raising and maintaining the blood pressure of dogs subjected to severe hemorrhage. These

facts suggest that alkali-treated bovine serum albumin may prove useful as a substitute for blood in man.

Note. Since submission of this paper for publication whole bovine serum, horse serum, and horse serum albumin have been treated with sodium hydroxide solutions. Such sera are not toxic and possess little or no antigenicity. Studies on the use of such alkali-treated sera for blood replacement in man and lower animals will be reported in later communications.

13760

Nitrites. X. Effect of Sodium Nitrite Upon the Blood Pressure of Unanesthetized Hypertensive Rats.*

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According to Grollman and his associates¹ nitrites and nitrates do not appreciably lower arterial blood pressure. An increase in cardiac output, they state compensates for the dilatation in the peripheral circulation and it is only when this stimulation of cardiac action fails that a fall in blood pressure ensues. It is maintained that in the case of the anesthetized animal the blood pressure decline is probably conditioned by this failure of the heart to respond owing to the disordered state of circulation induced by the anesthetic. In order to obviate any possible influence upon blood pressure, the experiments to be described presently were performed on the unanesthetized animal.

Measurement of Blood Pressure. The systolic blood pressures in the tails of rats were determined by the plethysmographic method of Williams, Harrison and Grollman.² The drug was not administered until a fairly constant level of blood pressure from day to day was established. The blood pressure of normal unoperated rats ranged

* The expense of this investigation has been defrayed in part by a grant from the Bressler Research Alumni Fund of the University of Maryland.

† The material contained in this paper is part of a thesis submitted by Maurice M. Rath to the Faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

¹ Grollman, A., Harrison, T. B., and Williams, J. R., Jr., *J. Pharm. and Exp. Therap.*, 1940, **64**, 76.

² Williams, J. R., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

between 75 and 110 mm of mercury, the mode was approximately 100. The variation from day to day for each rat, after sufficient training, was not more than 5 to 10 mm. In this experiment, each recording represents the average of 2 successive blood pressure readings varying in most cases not more than 0 to 5 mm.

Operative Technic. Rats were rendered hypertensive by (a) subtotal nephrectomy (Chanutin and Ferris³) or (b) bilateral wrapping of cellophane on the kidneys or (c) unilateral nephrectomy combined with unilateral cellophane wrapping, according to the method of Page.⁴ The last two procedures were found superior because the operation can be performed in 1 stage and a lower mortality rate results than with subtotal nephrectomy.

Only about one-half of the animals became hypertensive and it took from 2 to 6 months for hyperpiesis to develop. In the majority of hypertensive rats the duration of the elevated arterial tension

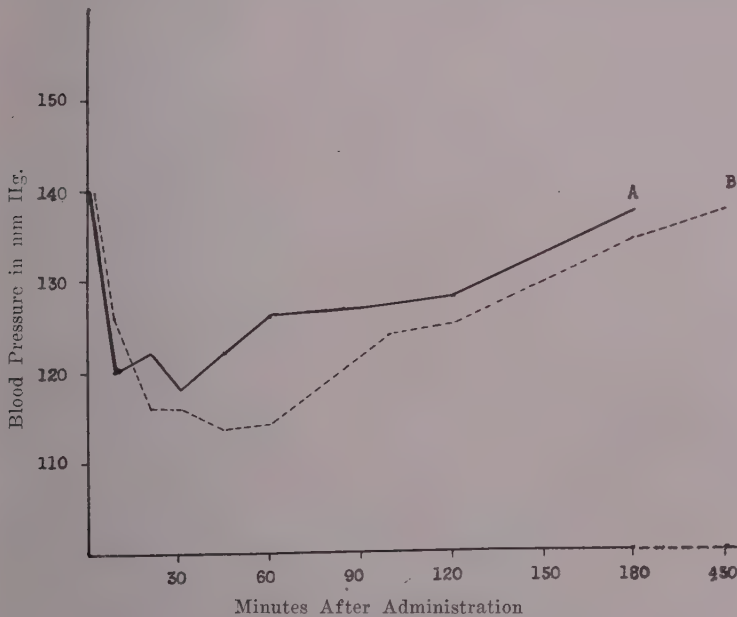


FIG. 1.

The Effect of Oral and Intraperitoneal Administration of Sodium Nitrite upon the Blood Pressure in Unanesthetized Hypertensive Rats.

Curves A and B show, respectively, the average fall in blood pressure after oral administration of 4 mg of sodium nitrite per 100 g and intraperitoneal injection of 2.5 mg of the same drug per 100 g body weight.

³ Chanutin, A., and Ferris, E. B., *Arch. Int. Med.*, 1932, **49**, 767.

⁴ Page, I. H., *J. Am. Med. Assn.*, 1939, **113**, 2046.

was limited; *i. e.*, about 6 months after the peak had been reached the blood pressure had fallen considerably.

Results of Administering Sodium Nitrite. Inspection of Fig. 1, which presents the results with a non-toxic, oral dose of 4 mg of sodium nitrite per 100 g of body weight, reveals that the blood pressure can be reduced by this drug. Twelve experiments were performed on 4 rats. The difference between the average blood pressure before oral administration (140 mm) and the lowest average blood pressure (118 mm, 30 minutes after feeding) was 22 mm of mercury. With the standard error of the difference equal to 3.1 mm, this difference is statistically significant.

Fig. 1 shows also the results of 12 experiments on the intraperitoneal injection of sodium nitrite (2.5 mg per 100 g of body weight) into 4 rats. An average reduction of 24 mm about 20 minutes after injection was observed. Statistical analysis shows that the standard error of the difference between the original blood pressure (140 mm) and the average blood pressure 20 minutes after injection (116 mm) is 6.4 mm.

No symptoms of nitrite poisoning were observed throughout the experiments. By oral administration, a dosage as high as 15 mg per 100 g was apparently not sufficient to evoke abnormal signs but 20 or more mg per 100 g was either toxic or lethal. Intraperitoneal doses of 10 mg or more were followed by toxic symptoms or death.

Grollman and coworkers¹ reported that inappreciable reductions in blood pressure were induced in hypertensive rats when sodium nitrite was given in a dosage of 0.1 g per rat per day mixed with the animal's food. Although this method of administration may effect a rather uniform and constant supply of the drug, it is conceivable that so little of the nitrite was ingested or available for absorption into the blood stream at any given time that a hypotensive action was not observed.

Summary. Sodium nitrite, when administered orally 4 mg per 100 g or intraperitoneally 2.5 mg per 100 g, reduces the arterial pressure of unanesthetized hypertensive rats.

13761

Riboflavin Absorption in Pernicious Anemia.

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Current opinion concerning the cause of the subacute combined cord degeneration in pernicious anemia favors the concept that the neural changes arise from a deficiency of some unidentified factor other than that required for hematopoiesis. Since achlorhydria invariably is present in pernicious anemia, the possibility must be considered that this condition might result in a decreased absorption of certain vitamins. That an avitaminosis might account for the neurological changes has been suggested by a number of observers among them being Field and his collaborators,^{1, 2} Gildea, Kattwinkel and Castle,³ Suh and Merritt,⁴ and others.

Street, Cowgill and Zimmerman⁵ observed myelin degeneration of the peripheral nerves and posterior columns of the spinal cord in dogs fed a diet adequate except for riboflavin. Their observations suggested the possibility that this component of the B complex may play a rôle in the etiology of subacute combined degeneration of the spinal column. In order to determine if pernicious anemia patients have a diminished ability to absorb this vitamin, its daily urinary excretion by such subjects was estimated.

Experimental. The experiment was conducted in a manner similar to that recently reported for pantothenic acid.⁶ Twenty-four hour urine specimens from 12 patients and 12 healthy students and laboratory workers, who served as control subjects, were collected and preserved with benzene and 10 ml of acetic acid. The patients obtained hospital meals, while the latter ate unrestricted diets likewise considered to be adequate. The method of Snell and Strong⁷ was used for the determination of the urinary riboflavin.

The values observed are recorded in Table I. Due to the unre-

¹ Field, H., Jr., Robinson, W. D., and Melnick, D., *Ann. Int. Med.*, 1940, **14**, 588.

² Robinson, W. D., Melnick, D., and Field, H., Jr., *J. Clin. Invest.*, 1940, **19**, 399.

³ Gildea, E. F., Kattwinkel, E. E., and Castle, W. B., *New England J. Med.*, 1930, **202**, 523.

⁴ Suh, T., and Merritt, H. H., *Am. J. M. Sc.*, 1938, **196**, 57.

⁵ Street, H. R., Cowgill, G. R., and Zimmerman, H. M., *J. Nutrition*, 1941, **22**, 7.

⁶ Meyer, C. E., Burton, I. F., and Sturgis, C. C., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **49**, 363.

⁷ Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, 1939, **11**, 346.

TABLE I.
Riboflavin Excretion.*

Pernicious anemia patients			Controls		
No.	Daily excretion,	After 5 mg dose,†	No.	Daily excretion,	After 5 mg dose,‡
	μg	μg		μg	μg
1	1070	3830	1	483	3360
2	15	2760	2	770	4750
3	250	1070‡	3	1200	2770
4	980	—	4	743	3080
5	825	2546	5	810	2180
6	980	4580	6	846	3180
7	—	4890	7	244	—
8	—	4410	8	312	4590
9	448	—	9	525	—
10	650	3530	10	615	2790
11	905	1600	11	1000	1890
12	716	1444	12	305	1700

*μg per 24 hours.

†5 mg riboflavin, Merek, given orally at start of 24-hour period.

‡An outpatient not available for further study.

stricted nature of the diets, of which the riboflavin contents were not known, the results cannot be interpreted in the nature of a comparative balance study. Nevertheless, they do serve to indicate that achlorhydria apparently does not impair the absorption of riboflavin.

More comparable data for the 2 groups of subjects were obtained by administering 5 mg of riboflavin in addition to that contained in the diets. Following this, the individual quantities excreted, listed in Table I, do not reveal any tendency toward a lesser output by the patients.

These results resemble those observed in a similar study of pantothenic acid excretion.⁶ They may be interpreted only as indicating that a lack of hydrochloric acid apparently does not result in an impairment of absorption of the accessory food factors studied. That the degeneration of the central nervous system may result from a conditioned avitaminosis arising from a lessened food consumption over a prolonged period remains a possibility.

Conclusions. In patients with pernicious anemia, the excretion of riboflavin, both before and after oral administration of 5 mg of the vitamin, was of the same order as that eliminated by healthy individuals. There is, thus, no indication of impaired absorption of this compound by such subjects as a result of their lack of hydrochloric acid.

13762

Chemotherapy of Chronic Progressive Arthritis of Mice. I. Rôle of Sulfur in Gold-Containing Compounds.

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(Introduced by M. H. Soule.)

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The apparent effectiveness of gold-containing compounds in the treatment of rheumatoid arthritis has stimulated interest in the mechanism by which these substances may act to relieve the symptoms of or arrest the course of this disease. Investigation of this problem is difficult because of the chronicity of the disease, lack of information concerning its etiology, and the inability to reproduce it in experimental animals. The infectious arthritis of mice described by Sabin¹ provides a tool by which certain aspects of the problem may be approached, since this experimental disease resembles rheumatoid arthritis in being definitely responsive to treatment with gold-containing compounds.

It seemed pertinent to determine whether compounds containing gold might be therapeutically effective because of properties other than those conferred by the presence of gold, especially since the undesirable effects which frequently accompany their use are believed to be due to the toxicity of this metal. Since most of the compounds used in therapy have contained sulfur in addition to gold, the question arose concerning the importance of this element, either for its own effect or in combination with gold. One of the more widely used compounds and a typical example of one containing both gold and sulfur is gold sodium thiomalate (Myochrysine). The configuration of the molecule of this compound (Table I) is such that one may conceive that the sulfur present might give rise to a sulfhydryl group on reduction. Because of the known importance of the sulfhydryl linkage in cellular physiology, it seemed possible that the therapeutic effectiveness of this and other gold- and sulfur-containing compounds might be due to the presence of this linkage in the molecule or its formation *in vivo*, rather than to the metallic moiety. The question as to the necessity of sulfur in any form was raised by Sabin² in his studies of the use of gold compounds in the therapy

* The Rackham Arthritis Research Unit is supported by the Horace H. Rackham School of Graduate Studies of the University of Michigan.

¹ Sabin, A. B., *Science*, 1939, **89**, 228.

TABLE I
 Structural Formulae of Compounds Used.

$ \begin{array}{c} \text{COONa} \\ \\ \text{H}-\text{C}-\text{S}-\text{Au} \\ \\ \text{H}-\text{C}-\text{H} \\ \\ \text{COONa} \end{array} $ <p>Gold sodium thiomalate</p>	$ \begin{array}{c} \text{COONa} \\ \\ \text{H}-\text{C}-\text{SH} \\ \\ \text{H}-\text{C}-\text{H} \\ \\ \text{COONa} \end{array} $ <p>Sodium thiomalate</p>
$ \begin{array}{ccc} \text{COONa} & & \text{COONa} \\ & & \\ \text{H}-\text{C}- & \text{S}-\text{S}- & \text{C}-\text{H} \\ & & \\ \text{H}-\text{C}-\text{H} & & \text{H}-\text{C}-\text{H} \\ & & \\ \text{COONa} & & \text{COONa} \end{array} $ <p>Disulfide sodium thiomalate</p>	$ \left[\text{Au}-\left[\begin{array}{c} \text{O} \\ \\ \text{C}-\text{CH}_2 \\ \\ \text{N} \\ \\ \text{C}-\text{CH}_2 \\ \\ \text{O} \end{array} \right] \right]^- \text{Na}^+ $ <p>Sodium succinimido-aurate</p>

of the mouse disease: he believed that results with gold chloride ruled out the need of this element. However, the gold chloride was highly toxic for the mice, and it was only on the basis of the cure of the few survivors of the therapy that this conclusion was made.

It is the purpose of this paper to report results of studies of the importance of the non-metallic portion of therapeutically effective gold- and sulfur-containing compounds and the need of sulfur in any form for the action of gold. We have confirmed previous work showing the effectiveness of gold sodium thiomalate in curing the mouse disease, and since this compound is commonly employed in human therapy, the use of sodium thiomalate and its disulfide form appeared to be a satisfactory means of investigating the importance of the sulfhydryl linkage. Additional study of the rôle of sulfur in therapy was possible by the use of sodium succinimido-aurate, a compound which, in contrast to most gold preparations used to treat arthritis, has the gold linked to the rest of the molecule through nitrogen, rather than through sulfur.

Methods. Two strains of pleuropneumonia-like microorganisms were used to infect mice. One of these was isolated from the lung of a rat showing "bronchiectatic" lesions described by Klieneberger and Steabben.² It produces a progressive arthritis in mice indistinguishable from that caused by Sabin's type B. (Serological studies with sera kindly furnished by Dr. Sabin have shown it to be similar to or identical with his type E strains obtained from mice. It resembles the "mouse type" strains of organisms in morphology,

² Sabin, A. B., *J. Bact.*, 1940, 40, 823.

³ Klieneberger, E., and Steabben, D. B., *J. Hyg.*, 1940, 40, 223.

and is apparently different from the L3 type which Klieneberger and Steabben found associated with the lung condition.) In addition, a type B strain obtained from Dr. Sabin was also used. We have found the course of this mouse disease to be essentially as described by Sabin.²

Therapy was begun one week after 50% of mice inoculated with 0.5 cc of 48-hour ascitic fluid-broth cultures of the organisms showed the presence of arthritis; this usually occurred within a week following injection. In each experiment, 40 mice were used; 3 groups of 10 mice each received treatment, and one group of 10 served as untreated controls; the mice in each treated group received a constant dose. Each group of treated mice received a different amount of one of the compounds (Table II) intravenously in 0.5 cc volumes on alternate days until 9 or 10 injections had been given, unless obvious cure had been obtained before. A therapeutic effect was considered to have been obtained when evidence of arthritis disappeared from at least 8 of the mice treated with one constant dose of the compound.

The compounds employed are listed in Table II. The sodium thiomalate was prepared by the method of Levene and Mikeska;⁴ the disulphide form was made by the oxidation of sodium thiomalate.^{5 †}

TABLE II.
Results of Chemotherapeutic Experiments.

Compound	Dose		Mice cured,* %
	Compound, mg	Gold content, mg	
Gold sodium thiomalate	1.0	0.5	60
	2.0	1.0	100
	4.0	2.0	100
Sodium thiomalate	1.54	0	0
	7.68	0	0
	15.40	0	0
Disulfide of sodium thiomalate	0.74	0	0
	3.84	0	0
	7.70	0	0
Sodium succinimido-aurate	0.86	0.25	40
	1.73	0.50	60
	8.65	2.50	100

*Average of several experiments with two strains of micro-organisms.

⁴ Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1924, **60**, 686.

⁵ Billmann, E., *Leibig's Ann.*, 1906, **348**, 120.

† Gold sodium thiomalate (Myochrysine) was obtained from Merck and Company. Sodium succinimido-aurate was obtained from Inventions, Inc., Chicago, Ill.

Results. The results of the experiments, summarized in Table II, emphasize the importance of gold in this type of therapy. Even when the amounts of sodium thiomalate and the disulphide form of sodium thiomalate containing the equivalent of 10 times the maximum amount of thiomalic acid contained in the largest dose of gold sodium thiomalate were administered, no therapeutic effect was observed. It is thus obvious that no part of the therapeutic effect of gold sodium thiomalate can be explained on the basis of the action of sulfur alone or on sulphydryl formation. In addition, the results with sodium succinimido-aurate rule out the need of sulfur in any form. These results demonstrate that, insofar as the mouse disease is concerned, the therapeutic effects are dependent on the presence of gold in the compounds employed. Further studies are being conducted with other heavy metals.

Conclusions. Arthritis of mice, caused by pleuropneumonia-like microorganisms has been treated with gold sodium thiomalate, sodium thiomalate, the disulphide form of sodium thiomalate and sodium succinimido-aurate. The compounds containing gold, gold sodium thiomalate and sodium succinimido-aurate, were effective in curing this disease, the others had no therapeutic effect. Sulfur alone or in sulphydryl linkage is not necessary in order that gold-containing compounds have a therapeutic effect.

13763 P

Changes in Plasma Amino Acid Nitrogen Concentration Following Nitrous Oxide and Ether Anesthesia and Surgery.

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The present observations were made as part of a general study of factors influencing the concentration of amino acids in the plasma. They are reported at this time because of the growing use of amino acid mixtures intravenously during surgical procedures.

Procedures. Nine patients were observed consecutively. The age, sex and operative procedure are noted in Table I. In each instance a blood specimen was obtained after an overnight fast and immediately prior to induction of anesthesia. Subsequently a second

blood specimen was obtained after induction of anesthesia with nitrous oxide and a third after satisfactory ether anesthesia was established and just before actual surgery was started, a fourth at the completion of the operative procedure, and a fifth 2 hours post-operatively. Loss of blood was negligible during the operations and no infusions were given during this time. Each blood specimen was drawn, transferred into a chilled paraffined tube, immediately centrifuged and the plasma removed. The plasma was then oxalated to prevent clotting and analyzed directly for alpha amino acids by the ninhydrin-CO₂ method¹ as modified for blood by MacFadyen, Van Slyke and Hamilton.²

Results. The results are summarized in Table I. In each patient a decrease in plasma amino acid nitrogen concentration occurred following induction of the anesthesia with nitrous oxide. In 5 patients no significant change in plasma amino acid nitrogen concentration was noted after the change from gas to ether anesthesia, while in four there was a slight decrease. At the conclusion of the operative procedure, the plasma amino acid nitrogen concentration had decreased further in all patients. Two hours following the operative procedure the plasma amino acid nitrogen concentration had again decreased in 5 of the patients while in 4 there was a moderate increase. The same pattern of change in plasma amino acid con-

TABLE I.
Changes in Plasma Amino Acid Nitrogen Concentration Following Anesthesia and Surgery.

No.	Age	Sex	Patient Operative procedure	Plasma amino acid nitrogen concn.				
				Pre-operatively mg%	After gas mg%	After ether mg%	After surgery mg%	Two hrs post-operatively mg%
1	15	F	Supracondylar Osteotomy, Right Femur	4.10	3.79	3.91	3.31	3.02
2	16	F	Triple Arthrodesis, Foot, Left	4.70	4.21	4.27	3.58	3.24
3	11	M	Triple Arthrodesis, Foot, Right	4.06	3.74	3.83	3.47	3.66
4	8	F	Shelf Operation, Hip, Left	4.46	4.27	4.27	3.62	3.83
5	10	M	Drainage of Psoas Abscess, Left	3.33	2.99	2.97	2.88	2.78
6	10	F	Sequestrectomy Saucerization, Right Femur	4.43	4.00	3.87	3.42	3.50
7	13	M	Rotary Osteotomy, Upper Third, Tibia, Right	4.60	4.25	4.06	3.58	3.47
8	11	M	Triple Arthrodesis, Foot, Right	4.46	4.08	4.01	3.48	3.32
9	15	F	Hallux Valgus, Operative Correction	4.94	4.62	4.51	4.15	4.42
Average				4.34	3.99	3.96	3.50	3.47

¹ Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, 1941, **141**, 627.

² MacFadyen, D. A., Van Slyke, D. D., and Hamilton, P., to be published.

centration was observed in the one patient with chronic hypoaminoacidemia (No. 5).

Discussion. The average plasma amino acid nitrogen concentration of this group of patients was 4.47 mg per 100 ml if the one patient with chronic hypoaminoacidemia is excluded. This value is in good agreement with the previously reported normal of 4.50 mg per 100 ml by this method.³ The observed decrease in plasma amino acid nitrogen concentration in the patients here reported was much greater than day to day fluctuations which we have observed in individual patients. Such fluctuations have remained within the remarkably narrow limits of $\pm .05$ mg per 100 ml plasma for periods as long as two months if there was no conspicuous change in the patient's condition.

Summary. In 9 children the concentration of amino acid nitrogen in the plasma decreased on the average of 0.38 mg per 100 ml following anesthesia with a further decline averaging 0.46 mg per 100 ml during a surgical procedure. In 5 of the 9, the plasma amino acid concentration was lower 2 hours after operation than at the conclusion of the operative procedure.

The pattern of change of plasma amino acid concentration in one patient with chronic hypoaminoacidemia was similar to those with normal concentrations preoperatively.

Additional studies are being prosecuted to elucidate an explanation of the factors causing the observed changes.

13764

The Portal Venous Pressure in Man.

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Mall^{1, 2} appears to have been the first to record the portal venous pressure in animals under experimental conditions. The later de-

³ Farr, L. E., McCarthy, W. C., and Francis, T., Jr., *Am. J. Med. Sc.*, 1942, **203**, 668.

* Now Captain in U. S. Army Medical Corps at O'Reilly General Hospital, Springfield, Mo.

¹ Mall, F., *Arch. f. Anat. u. Physiol.* (Du Bois-Reymond, *physiol. Abt.* 1892, p. 409).

² Mall, F. P., *Johns Hopkins Hospital Reports*, 1896, **1**, 111.

terminations by Burton-Opitz^{3, 4, 5} were shortly followed by Tigerstedt's⁶ records. These observers used the technically difficult T-cannulation of the portal vein, although this itself probably interfered with the normal pressure under investigation. In the experimental animal, we⁷ have employed cannulation of the divided inferior mesenteric vein, cannulation of a divided splenic vein (as suggested by Bayliss and Starling), and T-cannulation of the portal vein itself. The height of a column of physiologic saline solution above the portal vein sustained in a vertical tube attached to the cannula was taken as the portal pressure. In our animal, the portal pressure was always about 10 cm saline higher than the venous pressure simultaneously determined in the lower extremity. This communication is intended to record a series of portal venous pressures determined at laparotomy in man.

Rousselot⁸ measured the portal venous pressure in 4 patients with Bantis' syndrome of congestive splenomegaly without cirrhosis and observed the presence of portal hypertension—the pressures varying between 330 and 465 mm.

Method. A modification of the method of Griffith, Chamberlain, and Kitchell⁹ was used. The manometer consists essentially of a vertical glass tube with a T-cock at the lower end mounted on a firm base. One horizontal limb of the cock permits filling the manometer with saline to a level above the expected venous pressure. The other limb is attached by sterile rubber tubing to a glass adapter and hypodermic needle which are filled with saline solution. With the abdomen open, the needle is inserted into an omental vein by the surgeon, whereupon the T-cock is turned by the observer to allow the vertical manometer to communicate with the vein. The portal venous pressure is the vertical distance between the top of the sustained saline column and the portal vein. The same method was used to determine ankle venous pressure at the same time.

Results. The results in 17 cases are presented in Table I, from which it may be seen that in every one the portal venous pressure was higher than the ankle venous pressure. The average normal portal

³ Burton-Opitz, R., *Arch. f. d. ges. Physiol* (Pflüger), 1908, **124**, 469.

⁴ Burton-Opitz, R., *Quart. J. Exp. Physiol.*, 1912, **5**, 329.

⁵ Burton-Opitz, R., *Am. J. Physiol.*, 1914, **36**, 64.

⁶ Tigerstedt, R., *Ergebn. d. Physiol.*, 1920, **18**, 1.

⁷ Bellis, C. J., and Wangenstein, O. H., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 490.

⁸ Rousselot, L. M., *Surgery*, 1940, **8**, 34.

⁹ Griffith, G. C., Chamberlain, C. T., and Kitchell, J. R., *Am. J. Med. Sci.*, 1934, **187**, 371.

TABLE I.
Human Portal and Ankle Venous Pressures Determined Simultaneously at
Laparotomy (cm saline).

Case	Portal venous pressure	Ankle venous pressure	Difference
(a)			
1	18	9	9
2	17	8	9
3	21	10	11
4	16	6	10
5	20	7	13
6	17	9	8
7	14	7	7
8	18	5	13
9	20	11	9
10	17	9	8
11	21	12	9
12	19	6	13
13	22	11	11
14	15	8	7
15	18	7	11
16	17	9	8
			—
			Avg 10
(b)			
17	40	8	32
(portal cirrhosis)			

pressure was 10 cm saline higher than the average normal ankle venous pressure (Table I a). This value is what may be expected in order that the resistance in the portal capillaries may be overcome. In this series, normal portal pressures ranged between 14 and 22 cm saline. Normal ankle venous pressures ranged between 5 and 12 cm saline. In a case of advanced portal cirrhosis with ascites (Table I b), the portal venous pressure was 40 cm saline, and the ankle venous pressure 8 cm saline, a difference of 32 cm saline.

13765 P

Influence of Metrazol, Insulin Hypoglycemia, and Electrically Induced Convulsions on Reestablishment of Inhibited Conditioned Reflexes.

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In a series of studies it was shown that convulsions induced by metrazol and other convulsant agents or by electroshock as well as insulin hypoglycemia are accompanied by an increased excitability

* Aided by a grant from the John and Mary R. Markle Foundation.

of autonomic centers at the hypothalamic and medullary level.¹ It was thought that this action is of greatest importance for the shock therapy of psychoses and may explain the fact that these procedures have similar therapeutic effects although their action on the cortex as measured by the electroencephalogram is quite dissimilar.²

It seemed probable that hypoglycemic coma and convulsions may have in addition to their temporary influence on the cortex as revealed by the electroencephalogram far reaching effects on cortical processes which would be detected best by chronic experiments. Therefore the effects of these procedures on the conditioning process were studied.

The experiments were performed on 17 rats which received as an unconditioned stimulus a shock inducing them to jump from a compartment A across a small partition into the compartment B. This reaction was established by a few shocks. Thereafter a bell was sounded 2 seconds prior to shock. The sound continued during the shock. After the conditioned response (C.R.) was established at nearly 100% for 1 to 3 days the C.R. was inhibited by lack of reinforcement. When the C.R. response had either completely disappeared or was present only in 10-20% of the tests the rats were subjected either to metrazol convulsions, electroshock or insulin coma. Whereas control animals not subjected to these procedures showed no spontaneous recovery of the C.R. it was observed that the experimental group treated with coma or convulsions regained the inhibited C.R. in spite of continued lack of reinforcement. The duration of this effect was variable and depended on the number of "treatments" and in the case of insulin probably on the depth and the duration of the coma. After the effect had disappeared for several days the induction of a new coma or convulsions restored again the C.R. Fig. 1 gives a typical example for the effects of insulin, electroshock and metrazol. The fact that these procedures in spite of their different action on the electroencephalogram influence the C.R. in a similar manner suggests that increased autonomic discharges demonstrated in previous investigations under these

¹ Gellhorn, E., *Arch. Neurol. Psychiat.*, 1938, **40**, 125; *Am. J. Psychiat.*, 1941, **97**, 944, 1204; Gellhorn, E., Ingraham, R. C., and Moldavsky, L., *J. Neurophysiol.*, 1938, **1**, 301; Kraines, S. H., and Gellhorn, E., *Am. J. Psychiat.*, 1939, **95**, 1069; Gellhorn, E., and Darrow, C. W., *Arch. Inter. Pharmacodyn.*, 1939, **62**, 114; Yesnick, L., and Gellhorn, E., *Am. J. Physiol.*, 1939, **128**, 185; Domm, S., and Gellhorn, E., *Arch. Neurol. Psychiat.*, 1940, **43**, 726; Gellhorn, E., Kiely, W. F., and Hamilton, S. L., *Am. J. Physiol.*, 1940, **130**, 256; Gellhorn, E., Feldman, J., and Cortell, R., *Am. J. Physiol.*, 1940, **131**, 281; Gellhorn, E., and Kessler, M., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 64.

² Kessler and Gellhorn, unpublished observations on unanesthetized rats.

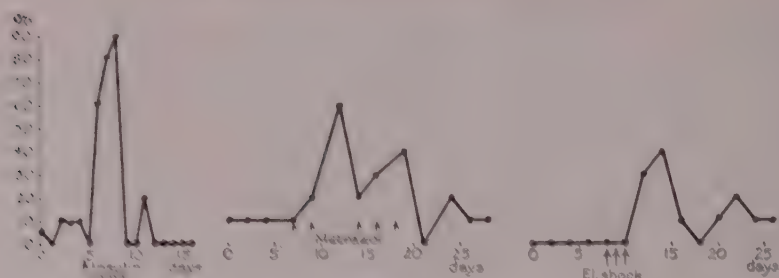


FIG. 1.

The effect of insulin coma, metrazol and electric shock on the restoration of inhibited conditioned reflexes. All rats were conditioned to the bell and after a conditioned reflex was established it was inhibited by lack of reinforcement. The records show that these inhibited reflexes are restored by insulin coma (5 units insulin per kilo intraperitoneally and 5 units subcutaneously), metrazol convulsions (45 mg per kilo) and electric shock applied to the head.

conditions may be responsible for these cortical effects. It appears to be significant for the understanding of the mechanism of shock therapy to point out that the procedures studied in this paper presumably act on the cortex by removing inhibitory processes. Rose and collaborators⁸ described a single sheep which for unknown reasons had lost its previously established C.R. and found that repeated insulin comas restored the C.R. The present work confirms and extends this observation, suggests the mechanism involved and indicates that convulsions as well as insulin coma may restore inhibited C.R.

13766

Antagonism of Anti-sulfonamide Effect of Methionine, and Enhancement of Bacteriostatic Action of Sulfonamide by Urea.*

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Holder and MacKay¹ have recently reported the use of a mixture of strong urea with sulfonamides topically applied to infected

⁸ Rose, J. A., Tainton-Pottberg, A., and Anderson, O. D., *Proc. Soc. Exp. Biol. and Med.*, 1928, **38**, 653.

* Supported by a grant from the General Research Fund of the University of Minnesota.

¹ Holder, H. G., and MacKay, E. M., *Military Surg.*, 1942, **90**, 509.

wounds. Urea markedly enhances the solubility of sulfonamides and could thus increase their action.

Urea offers certain advantages over other substances which have been described as having antisulfonamide inhibitor activity, such as azochloramid,^{2,3} and certain purines.⁴ Such inhibitors are methionine,⁵ para-amino benzoic acid, peptone, tissue extracts, purulent exudates, and so forth.⁶ These advantages are that urea is relatively nontoxic;⁷ it is a strong peptizing agent, exerting marked solvent action on necrotic tissue, pus and debris, and thus chemically debrides contaminated wounds and mechanically removes inhibitor; it lyses bacteria; it deodorizes foul smelling wounds rapidly; it renders sulfonamides more soluble; and it is very inexpensive. These properties have been reviewed elsewhere.¹

In addition, it would be highly important if an antisulfonamide-inhibitor action, or sulfonamide enhancing action could be demonstrated for urea.

Experimental. A strain of *Escherichia coli* was selected which acclimated itself to a synthetic medium of the following composition:

NH ₄ NO ₃	5.0 g
Na ₂ SO ₄	5.0 g
MgSO ₄	0.1 g
K ₂ HPO ₄	2.0 g
Glucose	10.0 g
Tap water to make volume to 1.0 liter.	

Glucose in itself, in concentrations of 0.6 to 2.6%, has been claimed to exert some antisulfonamide activity,⁸ although other reports have disagreed with this view.⁶ In our experiments this possible effect of glucose was controlled throughout by always using the same concentration and proper controls.

In this preliminary study, sodium sulfadiazine[†] was selected as the sulfonamide, and dl-methionine[‡] as the sulfonamide inhibitor.

Preliminary experiments were performed to find the tolerance range of *E. coli* in our basal medium to methionine and urea. Experi-

² Neter, E., *J. Pharm. and Exp. Therap.*, 1942, **74**, 52.

³ Schmuckes, F. C., and Wyss, O., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 232.

⁴ Harris, J. S., and Kohn, H. I., *J. Biol. Chem.*, 1941, **141**, 989.

⁵ Bliss, E. A., and Long, P. H., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 14.

⁶ Marshall, E. K., Jr., *Ann. Rev. Physiol.*, 1941, **3**, 643.

⁷ Olson, M., Slider, E., Clark, W. G., and MacDonald, R., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 396.

⁸ Hill, J. H., and Mann, E. F., *J. Urol.*, 1942, **47**, 522.

[†] Kindly supplied by Dr. Edward F. Roberts, of Lederle Laboratories, Inc.

[‡] Supplied through the courtesy of Dr. D. F. Robertson, of Merck and Co., Inc.

ments were also conducted to find that concentration of sodium sulfadiazine which would give partial bacteriostasis. Further inhibition was not desired because complete inhibition would conceal any enhancing effect of urea.

In concentrations below 1.8%, urea may act as an additional source of nitrogen for *E. coli*, but the experiments reported are not sufficient to demonstrate this possibility. 1.6% urea did not seem to show any marked effect on the growth of the test organism, and therefore, this concentration was used.

In concentrations between 1.0 to 100.0 mg %, methionine had no effect on the size of the populations in our medium, in 24 hours.

Sodium sulfadiazine was used in 1 mg % concentration in our tests because it caused an inhibition of 80% under the conditions of our experiments.

After autoclaving, the pH of the basal medium was approximately 7.0 as measured with a glass electrode. It was unaltered by the addition of the concentrations of sodium sulfadiazine, methionine and urea used.

Twenty-five ml amounts of medium containing the various test substances were inoculated with 0.1 ml of a 24-hour, synthetic medium culture of *E. coli* so that the initial populations at zero time were approximately 800,000 organisms per ml.

The numbers of bacteria after 24 hours of incubation at 37°C were determined by plating out in nutrient agar medium, five replicate plates per test culture. Counts were made after 48 hours of incubation at 37°C.

Results. The individual plate counts and the averages of the 5 replicate plates are given in Table I. All counts were made in a 1:10⁶ dilution of the original test cultures, except those so indicated. The counts are all expressed as 10⁶ organisms per ml of the test culture.

The above figures show that urea overcomes the inhibitory action of methionine on the growth inhibitory effect of sodium sulfadiazine on *E. coli*. Furthermore, urea in conjunction with sodium sulfadiazine displays greater growth inhibitory effect than the latter alone on *E. coli in vitro*.

Discussion and Conclusions. Whether the urea effect is an enhancement of the sodium sulfadiazine effect alone, or a neutralization of the methionine effect, or both, cannot be established from this preliminary data, although we are investigating this point further. However, under the conditions of our experiment, the following conclusions seem to be justified:

1. With the concentration of sodium sulfadiazine used, approximately 80% inhibition of growth of *E. coli* in synthetic medium occurred. 2. In the concentrations used, methionine and urea alone, or in combination, displayed no effect on the growth of the test organism. 3. Methionine almost completely inhibited the bacteriostatic effect of sodium sulfadiazine, thus confirming previous reports. 4. Urea and sodium sulfadiazine together showed a greater growth inhibitory effect than the latter alone. 5. Urea neutralized this inhibitory effect of methionine on sodium sulfadiazine and in conjunction with the sulfonamide, even though in the presence of methionine, displayed a greater growth inhibitory effect than the sulfadiazine alone.

Work is in progress to elucidate the action of urea on the effect of other inhibitors and on other sulfonamides. We are also investigating the action of urea on sulfonamide-fast pathogens in synthetic medium, and on the bacteriostatic action of sulfonamides in experimental and clinical treatment of infected wounds.

TABLE I.
Antagonism of Anti-sulfonamide-Inhibition of Methionine and Enhancement of Bacteriostatic Action of Sulfonamide by Urea.

Exp.	Control		SAD		M	U	U + M		U + SAD		M + SAD		U + SAD + M	
	1	2	1	2			1	2	1	2	1	2	1	2
I	193	231			231									
	207	238			214									
	170	252			242									
	191	249			229									
	183				226									
Avg	189	242			231									
II	186	223	48	40		230		*11.0	* 9.7	159	164	*19.9	*34.8	
	197	200	44	35		201		10.0	10.6	164	142	25.0	27.8	
	177	207	43	30		233		9.6	11.3	162	162	18.3	27.1	
	231	198	46	28		227		11.4	9.9	189	161	20.5	28.8	
	226	229	45	31		236		10.6	10.6	179	157	19.3	31.6	
Avg	203	211	45	33		225		10.5	10.4	171	157	20.6	30.0	
III	184	202	43	41		167	184	*14.6	*16.6	207	145	33	32	
	196	190	55	51		207	192	16.5	12.8	214	193	35	35	
	218	203	55	57		214	158	15.2	19.0	213	173	34	45	
	186	173	30	50		205	164	15.1	13.5	200	157	40	37	
	184	192	52	65		188	190	15.4	15.7	221	173	23	43	
Avg	193	192	46	53		196	177	15.3	15.5	211	168	33	38	

All figures represent 100 organisms per ml.

*Counts made on 10^{-5} dilutions. All others made on 10^{-6} dilutions.

SAD—synthetic medium with 1.0 mg% sodium sulfadiazine.

M—synthetic medium with 1.0 mg% methionine.

U—synthetic medium with 1.6% urea.

Clinical experience⁹ has confirmed the work of Holder and MacKay, and we feel that the beneficial action of urea in combination with the sulfonamides in the treatment of infections may be due partly to the effects described in this paper.

13767 P

Distribution of Vitamin A in Experimental Liver Damage.*

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The vitamin A content of cirrhotic livers is low^{1, 2, 3} but in acute human^{2, 4} and experimental⁵ hepatitis normal amounts are found. The blood vitamin A level in acute hepatitis, however, is low^{6, 7, 8} and hemeralopia appears.⁸ Histologically in human cirrhosis and hepatitis the vitamin A distribution is markedly altered; the total amount often reduced.⁹ To explain these alterations the histologic vitamin A distribution in experimental liver damage was studied.

Sixty-two rats were intoxicated with various doses of carbon tetrachloride. Of these 15 were on stock diet; 15 received supplements of 800 units of vitamin A every other day; 3 each received a high fat, high protein and high carbohydrate diet respectively. Of 23 vitamin A deficient rats, 13 received one dose of 800 units of vitamin A; another 6, 8 mg carotene. The organs were examined by routine histology and for vitamin A by fluorescence micro-

⁹ Strakosch, E. A., Clark, W. G., Tsuchiya, H. M., and Tenenber, D. J., manuscript in preparation.

* This work was supported by a grant from the Committee on Scientific Research of the American Medical Association.

¹ Moore, T., *Biochem. J.*, 1937, **31**, 155.

² Breusch, F., and Scalabrino, R., *Z. ges. exp. Med.*, 1934, **94**, 569.

³ Ralli, E. P., Papper, E., Paley, K., and Bauman, E., *Arch. Int. Med.*, 1941, **68**, 102.

⁴ Lindquist, T., *Act. Med. Scand.* (Suppl.), 1938, **97**, 1.

⁵ Lasch, F., *Klin. Wchschrft.*, 1935, **14**, 1070.

⁶ Lasch, F., *Klin. Wchschrft.*, 1938, **17**, 1107.

⁷ Breese, B. B., and McCoord, A. B., *J. Pediatr.*, 1940, **16**, 139.

⁸ Wohl, M. G., and Feldman, J. B., *Am. J. Digest. Dis.*, 1941, **8**, 464.

⁹ Popper, H., *Arch. Pathol.*, 1941, **31**, 766.

scopy.^{9, 10} Some livers were also chemically examined for vitamin A.¹¹

Within 24 hours after injection of 0.1 cc CCl_4 lipid droplets with strong vitamin A fluorescence accumulate in the center of the liver lobules. These develop by gradual enlargement of the small lipid droplets with vitamin A fluorescence which normally line the edge of liver cells. With repeated CCl_4 injections central necroses, proliferation and collapse of the connective tissue develop.¹² The central fat droplets become larger and their vitamin A fluorescence inhomogenous, the strongest fluorescence being in the immediate center. The vitamin A fluorescence disappears from the Kupffer and liver cells in the uninvolved peripheral areas and from the fatty changes of the intermediary zone. After administration of 5.6 cc CCl_4 in 27 injections within 69 days, a cirrhotic picture develops. The lobular pattern is reversed as the original centers are linked by proliferated connective tissue surrounded by liver cells with extensive fatty changes. Vitamin A fluorescence is imparted by only a few large fat droplets in the original center and by fine droplets in surrounding histiocytes. All other fat droplets which extend to the original periphery are free of fluorescence.

In rats receiving vitamin A supplements the fatty changes seem more extensive, but the connective tissue proliferation is less marked. Vitamin A fluorescence in the fatty areas is stronger, and the intact areas retain the fluorescence longer. In rats on high carbohydrate diet the fatty changes are less extensive than in those on high fat or high protein diet and the normal Kupffer and liver cells showed more fluorescence.

CCl_4 intoxication of vitamin A-deficient rats produced similar fatty changes as in normal rats but without vitamin A fluorescence of the fat. If these rats receive a dose of vitamin A or carotene, the large fat droplets in the center of the lobules acquire the fluorescence, whereas the histologically unaltered peripheral areas do not. Chemically less vitamin A is stored in the liver of the vitamin A-deficient rats with CCl_4 intoxication than in control deficient rats after feeding the vitamin. If both groups of rats are continued on vitamin A-deficient diet, the depletion of the smaller vitamin A stores in the large fat droplets of the intoxicated rats takes as long as that of the larger stores in the Kupffer and liver cells of the controls.

¹⁰ Popper, H., and Greenberg, R., *Arch. Path.*, 1941, **32**, 11.

¹¹ Josephs, H. W., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 112.

¹² Cameron, G. R., and Karunaratne, W. A. E., *J. Path.*, 1936, **42**, 1.

The change in the histologic vitamin A distribution during CCl_4 intoxication indicates a shift of vitamin A from morphologically intact peripheral areas of the lobules to the central fatty areas. This shift exceeds the altered fat distribution since small fat droplets in the normal periphery and medium sized ones in the intermediary zone are free of vitamin A fluorescence. It is problematic whether vitamin A actually moves from the periphery to the center, or whether it is more utilized from the periphery than from the central large fat droplets. The latter take up the vitamin faster and release it slower than the intact areas as shown by the repletion and depletion experiments respectively of vitamin A-deficient rats. This supports observations that in human liver damage the blood vitamin A level is low despite occasional quantitative normal liver stores,¹³ and that hemeralopia may occur. The fact that vitamin A supplements cause vitamin A storage in the intact areas after repleting the fatty ones, renders such therapy in liver damage rational. A high carbohydrate diet has a similar effect.

Intoxication of rats with phosphorus (15 rats), thyroxin (9) and bile acids (9) showed similar results.

Summary. In CCl_4 poisoning vitamin A is found in damaged but not in uninvolved liver areas. The pathologic areas take it up faster and release it slower than normal ones. High vitamin A therapy repletes the uninvolved areas.

13768 P

Sulfaguanidine in the Treatment of Infectious Enteritis in Swine.*

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The reports of Marshall, *et al.*,^{1, 2} on the use of sulfaguanidine in

¹³ Meyer, K. A., Popper, H., Steigmann, F., Walters, W. H., and Zevin, S., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 589.

* These studies were made possible with the assistance and supplies of sulfaguanidine by Dr. B. M. Lyon of Lederle Laboratories.

¹ Marshall, E. K., Bratton, A. C., White, H. J., and Litchfield, J. T., *Bull. Johns Hopkins Hosp.*, 1940, **67**, 163.

² Marshall, E. K., Bratton, A. C., Edwards, L. B., and Walker, E., *Bull. Johns Hopkins Hosp.*, 1941, **68**, 94.

TABLE I.
Sulfaguanidine in the Treatment of Infectious Enteritis of Swine.

Group No.	No. swine treated	No. control swine	Daily dose, g/kg	Results			
				Recovered No.	Recovered %	Deaths No.	Deaths %
1	52	31	.11-.40	36	69	16	31
				5	16	22	71
2	18		.07-.11	18			
	50		.11-.16	43		7	
	37		.16-.22	35		2	
	23		.22-.33	21		1	
	5		.33-.44	5			
	133			122	92	10	7.5
						1	.75

the treatment of enteric infections suggested a trial with this drug on infectious enteritis in swine. This disease, frequently referred to under such terms as necrotic enteritis or "necro", diphtheritic enteritis, infectious diarrhea, bloody scours, and Salmonellosis, is a widespread and important disease of swine. *Salmonella* types of organisms are usually considered as the principal causative agent.

Table I, Group 1, summarizes controlled experiments in 12 lots of pigs containing from 2 to 14 animals manifesting various forms of the disease (acute to chronic). The drug was administered in capsules and the daily dose divided between morning and evening. Group 2 represents several lots of animals treated under field conditions in which it was not possible to leave some as controls. Daily observations were made of them, however. The drug was administered in capsules or mixed with small amounts of feed.

In addition to the above experiments a total of 333 swine were treated under conditions of a general veterinary practice and with the coöperation of local practicing veterinarians. The results obtained were similar to those shown in Table I.

In general, the diarrhea was checked and the feces returned to normal consistency by the fourth or fifth day and the treatment was continued for an additional 3 or 4 days before the animals were released. A corresponding improvement in physical condition occurred.

These preliminary trials suggest that an effective dose is within the range of 0.165 to 0.33 g per kilo body weight (or 0.75 to 1.5 g/10 lb). In preliminary toxicity trials no evidence of ill effects was noted with dosages below 0.66 g/kg.

Blood Sugar Level and Influence of Hyperventilation on Slow Activity in Electroencephalogram.

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Interest has recently been focused on the relationship which seems to exist between slow potential changes appearing in the electroencephalogram (EEG) on hyperventilation and alterations in blood sugar level.^{1,2} If a direct relationship between the two does exist, it will be necessary systematically to control the blood sugar level in all hyperventilation studies. It is not easy to maintain such a control, and before the electroencephalographer becomes burdened with this precaution, it seemed desirable to investigate the possible relationship further.

Methods. One physically healthy, schizophrenic patient was observed on 10 different occasions* over a period of 10 weeks. Three glucose and 7 insulin (3, 5, 10 U) tolerance tests were given. Preceding the collection of each blood sample an EEG was obtained in the following manner: 5 meters of control record were followed by a 2-minute hyperventilation (at a regular rate of 30 deep respirations per minute), and the record allowed to return to its initial level. In this way, many hyperventilation records at various blood sugar levels were obtained.

Slow activity in the EEG was considered to include all waves with a frequency below 8 per second, and was qualified as total slow activity in the 7-meter period, which included the hyperventilation and recovery portions of the record.

Results. Fig. 1 A shows a definite inverse relationship between blood sugar and slow activity. Fig. 1 B, however, is typical of the remainder of our data. Here slow activity is inversely related to blood sugar only at 30 and at 90 minutes. In addition, it may be noted that the minimum amount of slow activity does not correspond with the maximum blood sugar value.

When all the blood sugar values obtained were plotted against the corresponding amounts of slow activity, an apparent relation-

¹ Davis, H., and Wallace, W. M., *Am. J. Physiol.*, 1941, **133**, 258P.

² Engel, G. L., and Margolin, S. G., *Arch. Neurol. Psychiat.*, 1941, **45**, 890.

* The patient was in the basal state on each occasion (fasting for at least 15 hours previous to the experimental session).

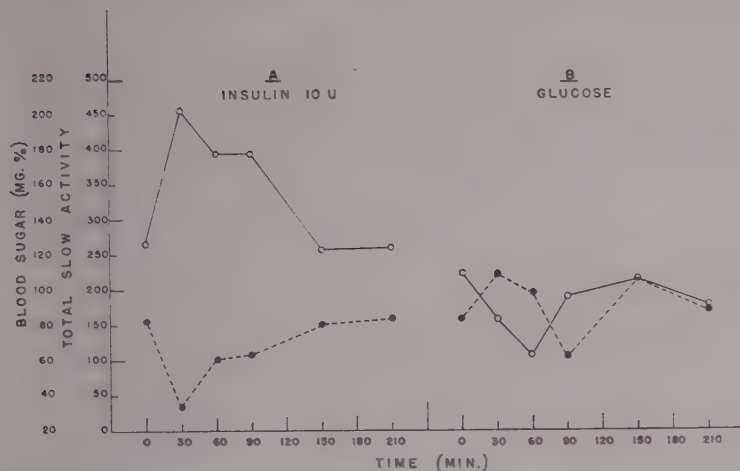


FIG. 1.

○ — ○ Total slow activity (forehead). Monopolar recording.
 ● - - - ● Blood sugar.

ship was found which held for blood sugars up to about 120 mg % (Fig. 2). This relationship, however, is only a rough trend, since the scatter of the data is great. For example, in individual instances blood sugars from 27 to 110 mg % all give corresponding total slow activity of about 220.

Discussion. It is apparent that decreasing blood sugar below 120 mg % alters the amount of slow activity appearing in the EEG

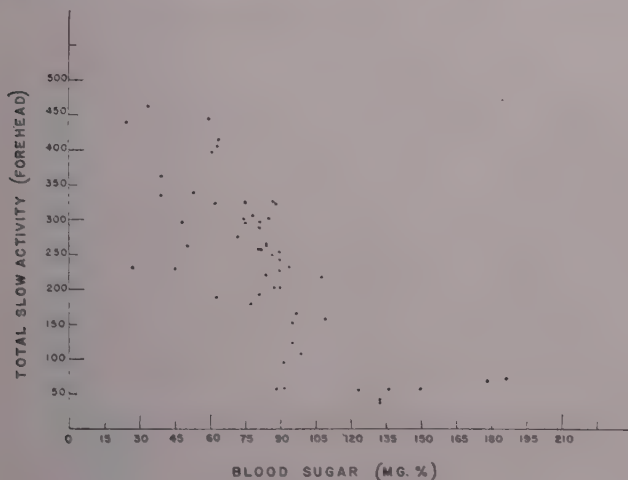


FIG. 2.

on hyperventilation. Our data show, however, that the relationship is not a simple one, since it is not possible to predict the amount of slow activity from the sugar value alone. As in all biological processes, there are many factors at work at the same time. In this case it is not at present possible to decide whether these factors cloud any direct relationship that sugar may have, or whether blood sugar level is not of primary importance but is capable of stimulating some other mechanism which controls the production of slow potentials in the EEG on hyperventilation. Until the other factors concerned with slow activity are identified, it is desirable to have the blood sugar as near as possible to 120 mg %.

It is doubtful that fasting is a highly significant variable in the appearance of slow rhythms after hyperventilation.³ In our experience, on some occasions fasting resulted in the appearance of slow waves on hyperventilation, but was without effect at other times.

Summary. Lowering blood sugar below 120 mg % may influence the response of the electroencephalogram to hyperventilation. Consequently it is advisable to keep blood sugar at a level of 120 mg % when the electroencephalogram is recorded during overventilation.

13770 P

Inhibition of Experimental Auricular Fibrillation by Procaine and Other Substances.*

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Shen and Simon¹ and van Dongen² have demonstrated that procaine (novocaine) inhibits the development of arrhythmias and ven-

³ Liberson, W. T., and Strauss, H., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 670.

* This work was carried on with the aid of a grant of funds from the Medical Research Fund of the Graduate School of the University of Minnesota.

Assistance in the preparation of these materials was furnished by the personnel of Works Projects Administration, Official Project No. 165-1-71-124, Sub-Project No. 335.

¹ Shen, T. C. R., and Simon, M. A., *Compt. rend. de la Soc. de Biol.*, 1938, **127**, 1457.

² van Dongen, K., *Arch. internat. de pharmacodyn. et de therap.*, 1936, **53**, 80; 1936, **54**, 252; 1938, **60**, 207.

tricular fibrillation which may occur upon the injection of epinephrine into cats and rabbits anesthetized with chloroform. Burstein, Marangoni and Rovinstine^{3, 4} have found that it exerts the same effect upon arrhythmias and ventricular fibrillation produced by epinephrine during cyclopropane anesthesia in dogs. Van Dongen² has shown also that procaine inhibits the development of both auricular and ventricular fibrillation from electrical stimulation of these chambers in cats and rabbits.

Since auricular fibrillation is much less persistent in cats and rabbits than it is in dogs and conditions in the latter approach more closely the clinical conditions in human beings we have investigated the effects of procaine and related substances on experimental auricular fibrillation in dogs.

All our animals were anesthetized with intraperitoneal injections of sodium pentobarbital (35 mg per kg).

Auricular fibrillation was produced in either of two ways: (1) Small fish hook electrodes were inserted into the walls of the right auricle and the auricle was stimulated for 5 seconds with the smallest stimulus from the secondary of a Harvard induction coil which would induce persistent fibrillation when the stimulus was repeated 3 times in rapid succession. The electrodes were made by soldering a very small fish hook to a piece of wire. This wire was passed through a piece of glass tube and then soldered to the end of a safety pin whose other end was fixed with sealing wax to the proximal end of the glass tube. The safety pin thus acted as a spring to clamp the fish hook tightly against the distal end of the glass tube. A piece of annunciator wire soldered to the safety pin connected the electrode with the pole of the induction coil. (2) We also produced auricular fibrillation by dropping acetyl beta methyl choline chloride solution on to the wall of the atrium by the method of Nahum and Hoff.⁵

A similarly constructed fish hook with glass tube and spring attachment was inserted in the wall of the right atrium. The lumen of this glass tube was 8 mm in diameter, and a small wad of cotton was fixed to its lower end so as to be in contact with the wall of the atrium. Five drops of a 1:500 solution of acetyl beta methyl choline chloride solution was then dropped into this tube and when it came

³ Burstein, C. L., and Marangoni, B. A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 210.

⁴ Marangoni, B. A., Burstein, C. L., and Rovinstine, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 594.

⁵ Nahum, L. H., and Hoff, H. E., *Am. J. Physiol.*, 1940, **129**, P 428.

into contact with the atrial muscle auricular fibrillation resulted. This fibrillation lasted from 11 to 12 minutes and could be reproduced with about the same duration.

In 5 dogs we have found that procaine in doses of 10 to 20 mg per kilo intravenously stopped fibrillation produced by the minimal effective faradic stimulation of the atria, but this resistance to fibrillation passed off rapidly, often within 5 minutes. Larger doses, 40 to 80 mg per kilo, prevented even maximal faradic stimulation from producing auricular fibrillation. In 4 dogs auricular fibrillation induced by dropping 5 drops of 1:500 solution of acetyl beta methyl choline chloride solution on to the atria was stopped within 35 seconds by the intravenous injection of 1 to 6 mg of procaine per kilo. The effect of the smaller doses of procaine was very transitory because the application of the same stimulus 5 minutes after cessation of the fibrillation usually caused the fibrillation to reappear.

At least 2 other local anesthetics seem to exert similar effects in inhibiting auricular fibrillation. In 3 dogs p-butyl amino-benzoyl-dimethyl amino-ethanol hydrochloride (pontocaine hydrochloride) in doses of from 0.7 to 12 mg per kg stopped auricular fibrillation produced by the minimal effective faradic stimuli, and in 2 dogs we obtained the same effect with 2-butyloxyquinoline carboxylic acid-4-diethylethylene diamide hydrochloride (nupercaine hydrochloride) in doses of from 1 to 3.75 mg per kg respectively.

Summary. At least three local anesthetics, procaine, pontocaine and nupercaine can inhibit experimental auricular fibrillation in dogs.

13771

Toxicity of Antiseptics for the Chick Embryo.*

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The resistance of the chick embryo to toxic substances is a subject which has been little explored¹ in spite of the fact that the embryo is well adapted to a variety of bacteriological and physiological experiments. The developing egg consists of rapidly growing em-

* This study was supported by the Virus Research Fund of the Lambert Pharmacal Company, St. Louis, Mo.

¹ Witlin, Bernard, *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 27.

TABLE I.
Lethal Effect of Injection of 0.05 cc of Antiseptic Agents into Incubated Eggs,
Experiments on Five Eggs in Each Lot.

Agent tested	Conc.	Death in each lot in 6 days			Mortality
Phenol	5.0%	4	2	4	10/15
	2.5%	2	1	1	4/15
	0.1%	0	0	0	0/15
Bichloride of Mercury	1:500	5	2	4	11/15
	1:1000	2	2	0	4/15
	1:10,000	1	0	0	1/15
Tincture of Iodine	25%	5	3	4	12/15
	1.0%	2	1	3	6/15
	0.1%	3	1	2	6/15
	0.01%	0	1	0	1/15
Copper Sulphate	5.0%	5	4	3	12/15
	0.1%	1	2	1	4/15
	0.01%	1	1	0	2/15
Potassium Permanganate	1:1000	0	2	1	3/15
	1:10,000	0	1	1	2/15
Ethyl Alcohol	25%	1	1	0	2/15
Propylene Glycol	100%	0	0	0	0/15
Boric Acid	4.0%	0	0	1	1/15
Boric Acid 2.5% in 25% Alcohol		0	0	0	0/15
Mild Silver Protein	10%	5	5	5	15/15
	5.0%	4	5	5	14/15
	1.0%	3	5	2	10/15
	0.1%	1	0	1	2/14
Liquor Antisepticus, N. F. VI	*	0	0	1	1/15
Listerine	*	1	0	1	2/15
Merthiolate	1:500	3	3	2	8/14
	1:1000	2	0	2	4/15
	1:10,000	0	2	0	2/15
Metaphen	1:500	4	4	2	10/15
	1:1000	2	1	0	3/15
Tincture of Metaphen	*	5	5	5	15/15
	1:10	3	2	3	8/14
	1:50	1	1	1	3/15
Mercurochrome	2.0%	3	3	4	10/15
	1.0%	1	1	2	4/15
	0.2%	0	1	0	1/14
Lysol	5.0%	0	1	1	2/15
Pot. Mercuric Iodide	1:5000	1	1	1	3/15

*Undiluted.

TABLE II.
Comparison Between Minimal Lethal Dose for Man and Chick Embryo.

Agent	Man		Chick embryo
	g/kg body wt according to		g/kg total egg wt
	McNally ²	Solis-Cohen, etc. ³	
Phenol	.014	.07	.05
Bichloride of mercury	.001	.002	.002
Iodine	.004	.019	.018

bryonic tissue in a perfect nutritional environment. It comes sealed in an ampule, the shell, which is readily opened for inoculation or other manipulation and easily sealed again for the period of re-incubation.

The original purpose of the present study was to determine whether the fertile egg could be used as a medium for testing the inactivating effect of antiseptics on viruses. Such studies could be made only if the normal physiology of the embryo is not greatly disturbed by the injection of such agents.

Three tests were performed on different days with each substance. Embryonated eggs which had been incubated for 6 or 7 days were candled and the location of the embryo marked on the shell. This mark was kept uppermost until the inoculation was completed. A hole was drilled through the shell over the air sac by means of a 2 mm dental bur or a carborundum disc. The injection was made by means of a 2-inch, 20 gauge needle fitted to a tuberculin syringe. The needle was directed slightly upward and inserted 3 cm into the egg. The amount injected was 0.05 cc in each instance. This represents 1 part in 1000 of total egg weight, or the equivalent of 70 cc for a man weighing 70 kilos. The eggs were candled daily and the day of death noted. The results are shown in Table I.

The minimal lethal dose for man has not been determined for many of these substances. In Table II the smallest amounts of phenol, bichloride of mercury, and iodine which cause death in man are expressed in grams per kilo of body weight. The corresponding figures for the chick embryo are also given, taking the minimal lethal dose as the amount which kills more than half but not all of the embryos. The similarity between the toxicity of these substances for man and for the chick embryo is striking.

² McNally, W. D., *Toxicology*, Industrial Medicine, Chicago, 1937, 108, 206, 812.

³ Solis-Cohen, S., and Githens, T. S., *Pharmacotherapeutics*, D. Appleton & Company, New York, 1928, 547, 671, 750.

13772

Activity of Penicillin *in vitro*.^{*}

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Fleming¹ observed that broth cultures of a certain strain of *Penicillium notatum* contained a substance, penicillin, which inhibited the growth of many Gram positive organisms. Stimulated by the work of Chain, *et al.*,² and Florey, *et al.*,³ who demonstrated the remarkable antibacterial action of impure preparations of penicillin, studies were carried out in this laboratory on its production and extraction and on its chemical and biological properties.^{4, 5, 6}

Method. Cultures of Fleming's strain of *P. notatum*¹ were grown for 3 to 5 days at room temperature on a honey agar medium. Saline suspensions of the spores from the cultures were seeded to 2-liter Erlenmeyer flasks containing 400 cc of a modified Czapek-Dox synthetic medium prepared according to the formula shown in Table I.

Brown sugar was used routinely in a concentration of 2% and

TABLE I.
Modified Czapek-Dox Medium.

	g
Sodium Nitrate (NaNO_3)	3.5
Potassium Phosphate (KH_2PO_4)	1.5
Potassium Chloride (KCl)	0.5
Magnesium Sulfate ($\text{MgSO}_4 + 7\text{H}_2\text{O}$)	0.5
Ferrous Sulfate ($\text{FeSO}_4 + 7\text{H}_2\text{O}$)	0.015
Brown Sugar (Jack Frost, dark)	20.0
Distilled H_2O to 1 liter	
Autoclave 15 lbs—15 min.	

* This work has been supported in part by a grant from the John and Mary Markle Foundation.

We are indebted to Dr. Martin H. Dawson for his valuable help and suggestions throughout this work.

1 Fleming, A., *Brit. J. Exp. Path.*, 1929, **10**, 226.

2 Chain, E., *et al.*, *Lancet*, 1940, **2**, 226.

3 Florey, H. W., *et al.*, *Lancet*, 1941, **2**, 177.

4 Dawson, M. H., Hobby, G. L., Meyer, K., and Chaffee, E., *J. Clin. Invest.*, 1941, **20**, 434.

5 Hobby, G. L., Meyer, K., Dawson, M. H., and Chaffee, E., *J. Bact.*, 1942, **43**, 11.

6 Meyer, K., *et al.*, *Science*, 1942, in press.

† Received through the kindness of Dr. Roger Reid, Johns Hopkins Hospital, Baltimore, Md.

gave a maximum titer of penicillin in 8 days. An equivalent concentration of dextrose required 12 to 14 days.

A temperature of 24°C proved optimal although good yields were obtained at various temperatures between 20° and 26°C.

White mycelial growth with or without early sporulation and accompanied by surface droplets was associated with high yields of penicillin. Excess sporulation was generally accompanied by low yields. There was no parallelism between the amount of yellow pigment produced in the medium and the amount of penicillin.

Titration of Activity. Two methods were used for the titration of the activity of penicillin: (1) the dilution method, and (2) the Oxford plate method.³

In the dilution method serial dilutions of penicillin in broth were seeded with a constant amount of a standard strain of hemolytic streptococcus (C203 Mv). For convenience failure to develop turbidity after 24 hours' incubation at 37°C was accepted as evidence of inhibition of growth. Activity was determined by measuring the least amount of penicillin necessary to inhibit growth of 2 to 4 million organisms. This method is rapid and relatively satisfactory if experimental conditions are kept constant.

The Oxford plate method as described by Florey, *et al.*,³ was employed.⁴ The method is based on preliminary dilutions and the error is therefore comparable with that of the dilution method. Certain additional disadvantages are inherent in the plate method. The size of the zone of inhibition is limited by the length of the lag phase of the test organism and by the depth and dryness of the agar. Thus the amount of activity measured is influenced by the extent to which diffusion of the test material has taken place before bacterial multiplication has begun.

Table II shows the results of titration of crude preparations of penicillin by the two methods. Within the limit of error, the two tests give comparable results.

TABLE II.
Titration of Penicillin.

Preparation	Dilution method, γ per cc	Oxford method, units per mg
NH II 2*	.4	42
98 A	.4	45
81 II	.8	25
90 D	.2	106
101 III	.08	240

*Oxford Standard Penicillin.

† Oxford penicillin NH II 2, having an activity of 42 units per mg, was used as standard. We are indebted to Dr. N. G. Heatley for supplying us with this material.

TABLE III.
Susceptibility of Organisms to Penicillin.

Susceptible strains	Insusceptible strains
<i>Pneumococcus</i>	<i>H. influenzae</i>
<i>Streptococcus hemolyticus</i>	<i>E. coli</i>
<i>Staphylococcus</i>	<i>B. typhosus</i>
<i>Meningococcus</i>	<i>B. dysenteriae</i>
<i>Gonococcus</i>	<i>B. proteus</i>
<i>Streptococcus viridans</i>	<i>B. paratyphosus</i> A
<i>B. subtilis</i>	<i>B. enteritidis</i>
<i>Cl. welchii</i>	<i>B. pyocyaneus</i>
<i>V. septique</i>	<i>B. fluorescens</i>
<i>Cl. histolyticus</i>	<i>B. prodigiosus</i>
<i>B. sporogenes</i>	Friedländer's bc.
<i>B. oedematis</i>	<i>Staphylococcus albus</i> —1 strain
<i>B. sordelli</i>	<i>Micrococcus albus</i> —1 strain
<i>Laetobacillus</i>	<i>Monilia albicans</i>
<i>Cryptococcus hominis</i>	<i>Monilia krusei</i>
	<i>Monilia candida</i>

Extraction. Penicillin was extracted from the acidified culture fluid with various organic solvents as described by Meyer, *et al.*⁶ It was obtained either as the salt or as the free acid.

Preparations of the ammonium salt were obtained routinely with an activity of 0.03-0.1 γ per cc against hemolytic streptococci. Crystalline preparations were obtained which also had an activity of 0.03 γ per cc, equivalent to 240 to 250 Oxford units per mg.

Activity in vitro. Penicillin was tested by the dilution method against a wide variety of organisms. Confirming the work of Florey and his coworkers,³ the preparations tested were found to exert a remarkable effect *in vitro* on many of the Gram positive organisms, both aerobic and anaerobic, as well as on gonococci and meningococci. The susceptible and insusceptible organisms tested are listed in Table III. Two strains of staphylococci were encountered which proved resistant.

All Gram negative organisms tested were relatively insusceptible to the action of this drug.

Considerable variation was found among different strains of the same organism as well as among the various organisms. The strains of pneumococcus and hemolytic streptococcus tested were 2 to 4 times more sensitive than staphylococcus, and 10 to 20 times more sensitive than some strains of non-hemolytic streptococci. (Table IV.) The effect was either bacteriostatic or bactericidal depending on experimental conditions.

The concentration of penicillin necessary to inhibit growth of or to kill Gram positive bacteria was found to be comparable to that of gramicidin and tyrocidin rather than to that of the sulfonamides.

TABLE IV.
Degree of Sensitivity of Various Organisms.

Organisms	Penicillin preparations Titer in γ per cc				
	NH II 2	101 III	7 II	No. 696	No. 924
<i>Streptococcus hemolyticus</i> (C203Mv)	.46	.08	.25	.12	.6
<i>Staphylococcus albus</i> (Oxford)	.93	.15			
<i>Streptococcus viridans</i> (Dup.)				.25	
Non-hemolytic <i>Streptococcus</i> (Cot)			250		
<i>Lactobacillus</i> (Leale)					2.5

Sterilization of a culture does not always take place with penicillin. Nevertheless there is always a marked decrease in the number of organisms present. Details will be presented in a subsequent paper. This is in sharp contrast to the effect produced by the sulfonamides which act only by decreasing the rate of multiplication.

Inhibition. Titrations of penicillin by the dilution method were carried out in the usual manner, using broth containing para amino-benzoic acid, blood, or serum. As reported by Florey, *et al.*,⁸ no inhibition by these substances could be demonstrated. Likewise, the titration of the activity of penicillin in Pappenheimer's synthetic medium⁷ containing no peptone indicated that peptone has no inhibiting action.

Confirming the work of Abraham and Chain,⁸ it was found that the supernatant broth from cultures *E. coli* does inhibit the activity of penicillin. The nature of this inhibitor will be discussed in another publication.

Summary. The observations of Florey, *et al.*,⁸ on the antibacterial activity of penicillin against Gram positive organisms are confirmed and extended. Preparations have been obtained of such activity that 0.03 γ inhibits the growth of 2 to 4 million hemolytic streptococci. This represents an equivalent of 240-250 Oxford units per mg.

⁷ Pappenheimer, A. M., Jr., and Hottle, G. A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 645.

⁸ Abraham, E. P., and Chain, E., *Nature, Lond.*, 1940, **146**, 837.

13773

Observations on the Mechanism of Action of Penicillin.*

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In previous reports^{1, 2} the preparation and *in vitro* activity of penicillin was described. Studies on its mode of action are herewith reported.

Rate of Killing. Pneumococci, hemolytic streptococci and staphylococci were tested with penicillin under various conditions, the number of surviving organisms per cc being determined at intervals. It was found that the number of survivors decreased by geometric units as time increased by arithmetic units. The log of the number of survivors plotted against time followed a straight line until at least 99% of the organisms were killed. Under the experimental conditions, pneumococci were destroyed at a more rapid rate than hemolytic streptococci, and hemolytic streptococci more rapidly than staphylococci. (Graph I.)

Furthermore, with a given concentration of penicillin the rate of killing of a given strain of hemolytic streptococci decreased as the number of organisms present at 0 hours increased. Likewise, with a given number of hemolytic streptococci the rate of killing increased within limits as the concentration of penicillin was increased. There was a point, however, beyond which, with a constant number of hemolytic streptococci per cc, increases in the concentration of penicillin no longer increased the rate of killing. (Graph II.)

The killing of hemolytic streptococci at a constant rate applied only to the destruction of approximately 99% of the organisms originally present. With the last 1% of the organisms, the reaction followed one of 3 courses: (1) The number of organisms continued to decrease at the same rate until complete sterilization had been effected; (2) the number decreased at a somewhat slower rate, or, (3) the number increased. It is therefore apparent that penicillin

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We are indebted to Dr. Martin H. Dawson for his valuable help and suggestions throughout this work.

¹ Hobby, G. L., Meyer, K., Chaffee, E., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 277.

² Meyer, K., *et al.*, *Science*, 1942, in press.

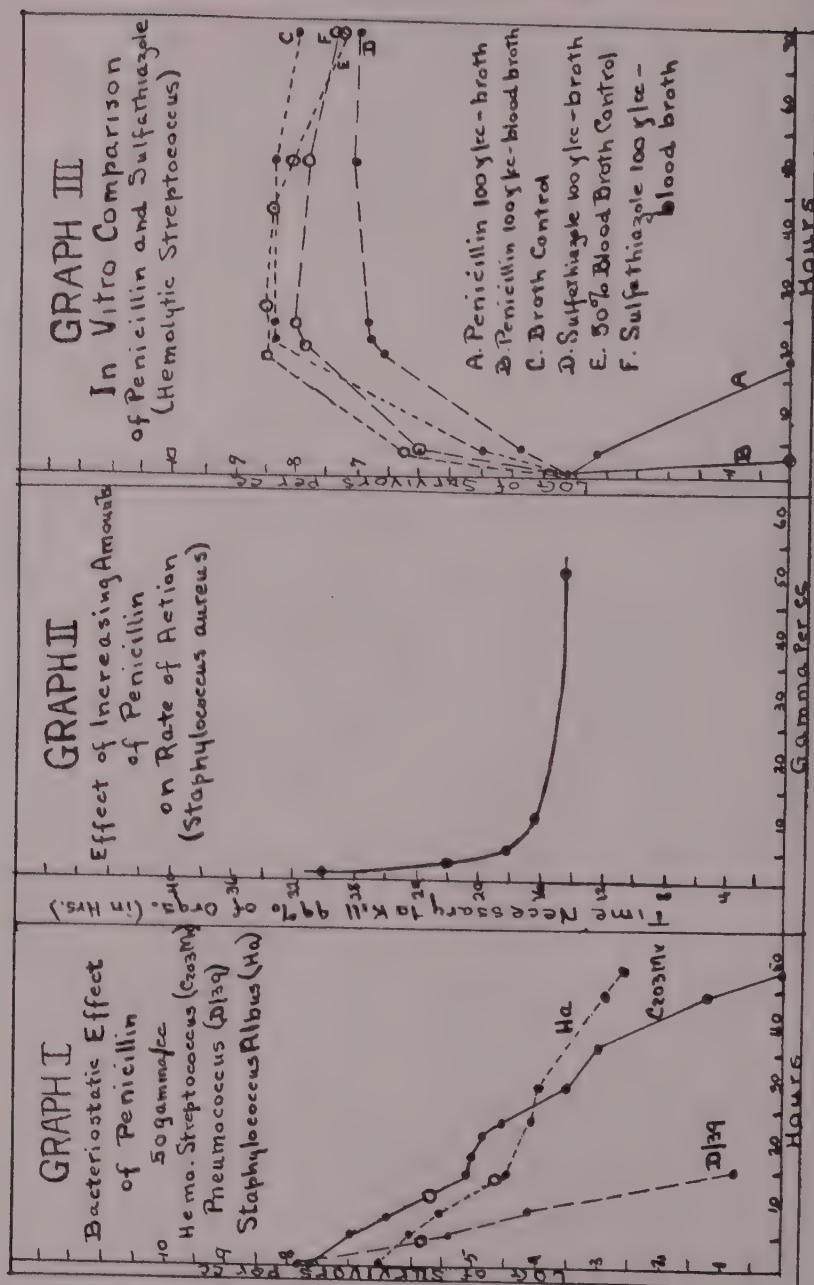


TABLE I.
Comparison of Penicillin, Gramicidin, and Tyrocidin *in Vitro*.

Culture	Inhibiting agent*	No. of viable organisms per cc $\times 1000$				
		0 hr	1 hr	3 hrs	7 hrs	24 hr
<i>Streptococcus hemolyticus</i> (C203 Mv)	Penicillin	1,500	4,300	2,650	420	0
	Gramicidin	1,500	2,430	1,140	7	2.4
	Tyrocidin	1,500	0.1	0	0	0

*10 γ per cc of each was used.

may act as a bacteriostatic agent or it may induce a true bactericidal effect.

The action of penicillin is in sharp contrast to that of the sulfonamides. Whereas penicillin produces an actual killing effect, the sulfonamides cause only a slowing up of the rate of growth. (Graph III.)

The activity of penicillin is comparable to that of gramicidin and tyrocidin. Penicillin, gramicidin, and tyrocidin all prevent multiplication and bring about a definite decrease in the number of organisms per cc. However, penicillin acts more slowly than either gramicidin or tyrocidin. (Table I.)

Lysis. To heavy suspensions of hemolytic streptococci, penicillin was added in concentrations as high as 100 γ per cc. No lysis of the organisms was observed on incubation at 37°C for as long as 24 hours.

Absorption. Broth containing penicillin was seeded with hemolytic streptococci (C203 Mv) in such a way as to give a final culture dilution of 10^{-2} . The cultures were incubated at 37°C and the number of organisms per cc counted at 0 and at 48 hours. At 0 and at 48 hours, portions were also filtered through a Seitz filter and the filtrates tested for penicillin activity. A definite decrease in the number of organisms per cc occurred, but no diminution in the amount of penicillin was detected. (Table II.)

In further experiments, 350 cc amounts of culture were centrifuged and the organisms suspended in 2 cc of broth. Four cc of

TABLE II.
Absorption of Penicillin by Bacteria. No. Organisms $\times 1000$.

No. of hours	Control Orgs. per cc	Penicillin	
		Orgs. per cc	Titer of supernatant
0	3,000	3,000	0.1 γ /cc
48	0	0	0.1 "
0	2,950,000	2,950,000	0.8 "
24	410	74	1.0 "

broth containing penicillin was added to 1 cc of this suspension, giving a final penicillin concentration of 340 γ per cc. Four cc of broth was added as control to 1 cc of the suspension of organisms. As in the previous experiment, the cultures were incubated at 37°C and the number of organisms per cc determined at intervals. At the same intervals portions were filtered and the filtrates tested for penicillin activity. Under these conditions the number of viable organisms remained unaltered and no loss of penicillin activity was observed. (Table II.)

Effect of Growth Phase on Activity of Penicillin. Undiluted 18-hour broth cultures of hemolytic streptococci (C203 Mv) were divided into 6 equal portions. To each of 3 samples, 0.1 cc of a solution of penicillin was added giving a final concentration of 100 γ per cc. To each of the remaining 3, 0.1 cc of broth was added. One tube from each set was incubated at 4°C, at 18°C, and at 37°C. The number of organisms per cc was determined at 0 and at 48 hours.

No multiplication took place in the control broth tubes either at 4°, 18°, or 37°C. At the same time no decrease in the number of organisms per cc occurred in the cultures to which penicillin was added although its activity remained unchanged. (Table III.)

Similar experiments were carried out using cultures containing fewer organisms per cc. These results are also illustrated in Table III. At 4°C no multiplication occurred in the control broth. At this temperature penicillin showed no bacteriostatic or bactericidal action on the organisms. At 18°C multiplication occurred at a slow rate and penicillin showed moderate antibacterial activity. At 37°C multiplication was rapid and the antibacterial action of penicillin was marked. It is apparent that penicillin is capable of destroying bacteria only if multiplication takes place. In similar experiments carbolic acid in a concentration of 0.1% was also effective only under conditions suitable for multiplication.

Summary. Penicillin acts either as a bacteriostatic or bactericidal

TABLE III.
Effect of Temperature and Concentration of Organisms on Penicillin Activity.
No. of Organisms \times 1000.

Medium	Organisms per cc at 0 hr	Organisms per cc at 48 hours		
		4°C	18°C	37°C
Broth penicillin, 100 γ /cc	5,500 5,500	6,480 5,280	17,000 320	130,000 0.1
Broth penicillin, 100 γ /cc	94,000 94,000	90,000 92,000	67,500 60,000	85,000 126,000

agent depending on the experimental conditions. The number of organisms decreases at a constant rate until 99% of the organisms have been destroyed. The rate of killing varies with different organisms. The action of penicillin on hemolytic streptococci is not accompanied by lysis of the organisms. No detectable amount of penicillin is destroyed or absorbed from solution by the organisms. It appears to be effective only when active multiplication takes place.

13774

Chemotherapeutic Activity of Penicillin.*

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The activity of penicillin *in vitro* has been discussed in detail in previous communications.¹ Its *in vivo* activity was first reported by Chain, *et al.*,² and by Florey, *et al.*³

In vivo Activity. Mice infected intraperitoneally with varying amounts of a highly virulent strain of hemolytic streptococci (C203 Mv) were treated subcutaneously with small amounts of penicillin.

TABLE I.
Effect of Subcutaneous Injection of Penicillin on Group A Hemolytic Streptococcus Infections (C203 Mv).

Dilu. of culture	No. of orgs. inj. $\times 1000$	No. of mice	Amt. of penicillin,* cc	No. of days treated	No. dead (<48 hrs)	No. prolonged life (2-7 days)	No. survived (>7 days)
.2.0cc	180,000	9	.4-.65	<6	3	1	5
1.0cc	90,000	10	.4-.60	<6	1	2	7
10-1	9,000	15	.2-.58	<6	2	4	9
10-2	900	13	.2-.57	<6	0	4	9
10-3	90	11	.2-.57	<6	0	3	8
Controls							
10-5	0.9	15	0	0	15	0	0

*Alcoholic solution: Total dosage per mouse calculated to be <7 mg dry weight.

* This work has been supported in part by a grant from the John and Mary Markle Foundation.

We are indebted to Dr. Martin H. Dawson for his valuable help and suggestions throughout this work.

¹ Hobby, G. L., Meyer, K., Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, 50, 277, 281.

² Chain, E., *et al.*, *Lancet*, 1940, 2, 226.

³ Florey, H. W., *et al.*, *Lancet*, 1941, 2, 177.

TABLE II.
 Titration of Activity of Penicillin* Against Hemolytic Streptococci *in Vivo*.

Dilution of culture	Penicillin (mg)	No. mice	No. survived
10 ⁻²	1.5	3	3
	1.0	3	3
	0.75	3	3
10 ⁻¹	1.5	3	3
	1.0	3	3
	0.75	3	0†
Undiluted	1.5	4	4
	1.0	4	2†
Controls			
10 ⁻⁶	0	10	d. < 2 days
10 ⁻⁷	0	10	d. < 2 days

*Ammonium salt containing 150-200 Oxford Units per mg.

†Two mice showed prolonged survival time in each instance.

Table I shows the results of treatment with crude preparations, and Table II shows the results with highly purified material. It is apparent that 1.5 mg of penicillin is sufficient to protect mice against 1 cc of whole culture containing at least 1,000,000 lethal doses. Amounts as small as 0.75 mg protected against 10⁻² dilutions of culture containing at least 1000 lethal doses. Treatment was effective if started as long as 8 hours after infection. (Table III.)

Mice were infected similarly with varying amounts of a highly virulent strain of pneumococcus type II. The subcutaneous injection of similar amounts of penicillin again gave adequate protection.

Further experiments showed penicillin to be effective intraperitoneally and intravenously as well as subcutaneously.

Penicillin is rapidly excreted when injected in solution and frequent injections are therefore necessary. This difficulty was eliminated by the use of suspensions in oil or dry pellets.

Oil Suspensions. Penicillin, prepared in the form of the sodium salt,¹ was dissolved in a drop of water and the solution mixed with

 TABLE III.
 Subcutaneous Injection of Penicillin Started 8 Hours after Intraperitoneal Injection with Hemolytic Streptococci: Strain C203Mv.

Dilu. of culture	No. of mice	Amt. penicillin,* cc	No. of days treated	No. died† (<48 hrs)	No. prolonged life (2-7 days)	No. survived (>7 days)
10 ⁻⁵	3	.48	4			3
10 ⁻⁶	3	.51	4	1	1‡	1
10 ⁻⁷	3	.48	4		1‡	2

*Alcoholic solution: Total dosage per mouse calculated to be <7 mg dry weight.

†5 untreated control mice died in <30 hours.

‡Hearts' blood cultures taken at autopsy showed no organisms.

† For part of the sodium salt used we are indebted to Chas. Pfizer and Co., Brooklyn, N.Y.

TABLE IV.
Effect of Subcutaneous Injection of Oil Suspensions of Penicillin on Hemolytic Streptococcus Infections in Mice.

Dilution of culture	No. of mice	Penicillin, mg	No. died (<48 hrs)	No. prolonged life (2-7 days)	No. survived (>7 days)
10-1	6	3.0		3	3
10-3	24	3.0-4.0	7	3	14
Controls					
10-6	9		9		
10-7	9		9		

sesame oil. A single subcutaneous injection of approximately 3.0 mg of penicillin in such a suspension was adequate to protect 66% of mice against the intraperitoneal inoculation of over 1000 lethal doses of hemolytic streptococci. (Table IV.)

Dry Pellets. Penicillin was mixed with 4 parts of pure cholesterol and ground to form a homogeneous mixture. Pellets of the mixture containing 1.5 to 3 mg of penicillin each were prepared in a pill press.

The implantation of a single pellet subcutaneously, 1 to 4 hours after infection, protected 66% of mice against over 1000 lethal doses of hemolytic streptococci (C203 Mv). (Table V.)

Toxicity. Mice were injected intravenously with varying amounts of penicillin in solution. The LD₅₀ for an 18 g mouse was found to be 32 mg of the sodium salt or 12 mg of the ammonium salt—equivalent to 1.8 g and 0.67 g respectively per kg body weight.

With lethal doses no abnormal pathology was observed other than congestion of the lungs. Immediate choking and gasping followed the injection and death occurred within 1 to 3 minutes. With sub-lethal doses, respiration was extremely rapid, followed by complete prostration lasting for a variable length of time. Recovery followed after a period of between 15 minutes and several hours.

Guinea pigs were inoculated intravenously with 320 mg of the sodium salt of penicillin or the equivalent of 1.3 g per kg of body

TABLE V.
Effect of Subcutaneous Implantation of Dry Penicillin on Hemolytic Streptococcal Infections in Mice.

Dilution of culture	No. of mice	Penicillin, mg	No. died (<48 hrs)	No. prolonged life (2-7 days)	No. survived (>7 days)
10-3	29	1.5-3.0		8	21
Controls					
10-6	6	0	6		
10-7	6	0	6		

weight. No pyrogenic or other toxic symptoms were observed.

The toxicity of the ammonium salt was tested by Dr. Phillips Thygeson in tissue culture, in the chorioallantoic membrane, and when applied directly to the human eye.⁴ No toxicity was observed.

Sodium and ammonium salts of penicillin were administered to humans. The daily injection of 170 mg of purified penicillin, having an activity of 240 Oxford units per mg, for a period of 6 days produced no untoward effect.

Summary. Penicillin is highly effective against hemolytic streptococcus and pneumococcus infections in mice when given subcutaneously, intravenously or intraperitoneally. When given in the form of oil suspensions or dry pellets, a single subcutaneous injection confers a high degree of protection. Penicillin is apparently non-toxic within the range of therapeutic dosage.

13775 P

Effects on Arterial Hypertension of Heat-inactivated Tyrosinase Preparations.*

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Recent studies of the mechanisms involved in the production of experimental renal hypertension show that several humoral agents may be involved. Following the hypothesis that phenolic amines may be involved in renal hypertension, Schroeder and Adams¹ found tyrosinase preparations from mushrooms to be effective in lowering the blood pressures of hypertensive animals. Their studies were extended to the effects of tyrosinase preparations on arterial hypertension in man and reported results in 17 patients, with significant falls in blood pressures in 13 of these following daily administration of unspecified amounts of their tyrosinase preparations. Some

⁴ Thygeson, P., personal communication.

* Aided by grants from the Beaumont Trust Fund and the Dazian Foundation for Medical Research.

[†] Glick Fellow in Cardiovascular Research.

[‡] Tom May Fellow in Cardiovascular Research.

¹ Schroeder and Adams, *J. Exp. Med.*, 1941, **73**, 531.

phenolic substance was considered to be altered by the injected enzyme to account for the lowered blood pressures observed.

The present experiments were carried out with mushroom tyrosinase preparations made by modifications of the purification procedures of Keilin and Mann.³ The valuation of the enzymic activity was made upon a catechol-hydroquinone substrate in terms of "catecholase" units as defined by Adams and Nelson.⁴ The final preparations were made up to contain 500 "catecholase" units per ml and contained about 2 mg per ml of non-dialyzable total solids. By heating such preparations for 40 minutes to 60 °C, about 95% of the "catecholase" activity was destroyed, and such preparations are referred to as heat-inactivated.

Four patients with malignant or premalignant hypertension were hospitalized and observed before treatment for 7 to 10 days, with blood pressures taken twice or more daily. In the first patient studied, the marked local reactions from injections of the unheated enzyme preparations suggested that the hypotensive response might be due to non-enzymic substances in the preparation. Treatment was then continued with heat-inactivated preparations and it was observed that, while the enzymic activity was almost completely absent and the local reactions were somewhat less, these preparations were still very effective in lowering blood pressure. The 3 subsequent patients were consequently only treated with the heat-inactivated preparations, and there also resulted marked falls in their blood pressures. Two of the patients showed falls in blood pressure from control levels of 210/160 and 210/150 to 170/130 and 135/90 respectively, when 5 ml intramuscularly was being injected daily during the following treatment period. In these 2 patients the T-waves of their electrocardiograms became upright and their cardiac size decreased. Papilledema and hemorrhages of the eye grounds regressed or disappeared and the patients felt better subjectively. The subject whose record is shown in Fig. 1 was almost blinded by hypertensive retinopathy, but after the tenth injection could read a newspaper. On Fig. 1 are charted the blood pressure and temperature observations made on this patient. In the other 2 patients the dosage was not uniform throughout the treatment period, but dosages of from 3 to 8 ml were given with resultant lowering of blood pressure from control levels of 220/150 and 200/110 to 178/128 and 150/90 respectively.

² Schroeder, *Science*, 1941, **93**, 116.

³ Keilin and Mann, *Proc. Roy. Soc. London B*, 1938, **125**, 187.

⁴ Adams and Nelson, *J. Am. Chem. Soc.*, 1938, **60**, 2474.

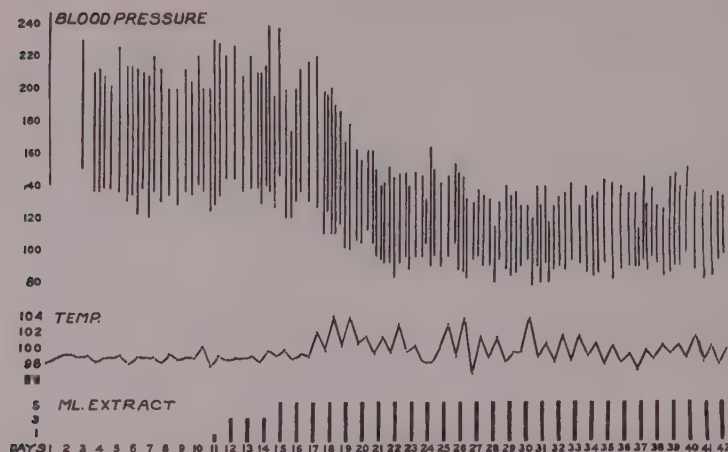


FIG. 1.

Effects of intramuscular injections of from 1 to 5 ml of heat-inactivated tyrosinase preparations 54K and 58K on arterial blood pressure and temperature of patient with arterial hypertension.

Effects other than upon the symptoms of arterial hypertension are notable. Local inflammatory response and systemic reactions such as chills and fever of varying degree, perspiration, with general malaise and rarely, nausea, are observed. Such effects have also been notable in the therapeutic experiments with kidney extracts that have been reported by Page and coworkers.⁵ These other effects, noted both with mushroom and kidney extracts, resemble those which may be expected after injection of a non-specific protein material, and it seems possible that such effects and the therapeutic results so far observed are closely related.

The observations demonstrate that heat-inactivated tyrosinase preparations can produce significant lowering of blood pressure and remission of other symptoms of arterial hypertension in man. Such effects as have been observed are as marked as those which have been reported by others following injections of active tyrosinase preparations, and therefore show the effects upon the symptoms of arterial hypertension to be unrelated to the enzyme content of the preparations.

⁵ Page, Helmer, Kohlstaedt, Kempf, Gambill and Taylor, *Ann. Int. Med.*, 1941, **15**, 347.

13776

Occurrence of Psittacosis-like Infection in Domestic and Game Birds of Michigan.*

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The latent presence of a form of psittacosis in pigeons, called by Meyer, ornithosis, has been demonstrated by Pinkerton and Swank,¹ Coles,² and Meyer, Eddie and Yanamura.³ The characteristics of the virus indicate that it is related to the virus of typical psittacosis but resembles more closely the type of psittacosis virus found in Australian parakeets.⁴ Moreover, available evidence points to the similarity of its pathogenic characteristics and those of the virus of meningo-pneumonitis (M-P) originally described by Francis and Magill.⁵ Recent serological studies have clearly shown that a close relationship exists between the viruses of psittacosis, meningo-pneumonitis and lymphogranuloma venereum^{6, 7} and that patients infected with one of the viruses produce antibodies which fix complement in the presence of any one of the 3 antigens.

That the virus of pigeon origin may infect human subjects is shown by actual recovery of the virus from patients,⁶ some of whom were intimately associated with infected birds.⁸ These results have heightened the interest in the pigeon as a disseminator of disease and the recent report of Meyer, Eddie, and Yanamura³ has furnished evidence of the wide distribution of infection in this species of bird. Studying pigeons from California, New York, Indiana, Iowa, and South Carolina, they found that the sera from approximately 50% of the 237 pigeons tested fixed complement to some extent in the presence of psittacosis antigen and from 25 of the pigeons virus of ornithosis was actually recovered.

* This study was conducted under a grant from the International Health Division of the Rockefeller Foundation.

¹ Pinkerton, H., and Swank, R. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **45**, 704.

² Coles, J. D. W. A., *Onderstepoort J. Vet. Sci. and An. Ind.*, 1940, **15**, 141.

³ Meyer, K. F., Eddie, B., and Yanamura, H. Y., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **49**, 609.

⁴ Meyer, K. F., and Eddie, B., *J. Inf. Dis.*, 1939, **65**, 234.

⁵ Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, **68**, 147.

⁶ Eaton, M. D., Beck, M. D., and Pearson, H. E., *J. Exp. Med.*, 1941, **73**, 641.

⁷ Rake, G., Eaton, M. D., and Shaffer, M. F., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **48**, 528.

In view of this evidence the present study was undertaken to ascertain the distribution of ornithosis in the avian species of Michigan.

1. *Complement-fixation Test.* (a) *Preparation of Antigen.* In this study the antigen was prepared in 100 cc amounts, a total of 1100 mouse lungs being used. Fifty mice were inoculated intranasally with 0.05 cc of a 10% lung-suspension of the highly virulent F97 strain of meningo-pneumonitis virus. After 45 to 48 hours the lungs were removed from the mice aseptically and ground in a mortar with physiological saline (pH 7.6), approximating 2 cc of saline per lung. The procedure followed thereafter was the same as that employed in the preparation of psittacosis antigen.⁸ Exactly the same procedure was followed in the preparation of antigens from normal mouse lungs which were used for controls.

(b) *Serum* was obtained from blood taken aseptically from the ulnar vein of the wing or if the bird was to be sacrificed, blood was taken from the heart.

(c) The test was performed in essentially the same manner employed for the diagnosis of psittacosis.⁸ Two *full* units of complement were used in the tests here reported. The hemolytic system consisted of washed sheep cells suspended in a dilution of amboceptor so that 0.5 cc contained 2 units of amboceptor and a 2% suspension of red blood cells. The end point adopted was the highest dilution in which complement was completely fixed.

As a rule, each serum was titrated against both infected and normal lung-antigen. Owing to the fact that human sera give cross reactions with the antigen of lymphogranuloma venereum, a certain number of tests were made with the latter antigen as well.

2. *Isolation of Virus.* In each instance, groups of 3 or 4 mice were inoculated intraabdominally with 10% suspensions of liver and spleen, kidneys and ovaries or testes, and 1% intestinal contents, respectively, from a single pigeon. The mice were kept in isolated quarters. After an interval of 21 to 30 days all surviving mice were sacrificed and passage was made with suspensions of liver and spleen intranasally to normal mice. After a similar interval an additional passage with suspensions of the lungs of these mice was made intranasally. If no abnormalities were detected after 3 transfers, the series was discontinued. If pulmonary lesions were found the passages were continued until virulence was enhanced. Impression-smears from the lungs were made, stained by the Machiavello

⁸ Meyer, K. F., and Eddie, B., *J. Inf. Dis.*, 1941, **68**, 1.

technic⁹ and examined for elementary bodies. The characteristic pulmonary pathology also served in identification of the agent.

The tests were controlled by the simultaneous maintenance of mice inoculated with non-infectious material under the same conditions.

Results. (a) *Pigeons.* The pigeons were obtained as follows: 24 from the Detroit Market; 24 from loft J. S., 12 from loft J. C., Ann Arbor; 20 from loft K in East Lansing; 18 from loft Dr in Lansing. The results of the complement-fixation tests are summarized in Table I. It is seen that positive reactions were obtained with 61% of the sera. The general incidence corresponds with that reported by Meyer, Eddie, and Yanamura³ in other states. The percentage of positive reactions among the specimens from different groups ranged from 8.5 to 85. No significant difference in the number of reactors could be attributed to sex, age, or the size of the pigeon's spleen.

It is of interest to record that while a marked parallel is obtained in the reaction of the serum of human subjects to the viruses of meningo-pneumonitis, psittacosis, and lymphogranuloma venereum, pigeon serum exhibits a greater specificity. The reaction to antigen of typical psittacosis was essentially the same as to M-P antigen.

TABLE I.
Complement-Fixation Test with Serum of Pigeons in Michigan.

Titer	Detroit Market 1-13-42	Ann Arbor Loft J.S. 12-24-41	Ann Arbor Loft J.C. 1-9-42	Ann Arbor Loft J.S. 4-2-42	E. Lansing Loft K. 3-26-42	Lansing Loft Dr. 3-26-42	Total
0	9	3	8	5	3	5	33
1:2	1	1	1	0	1	1	5
1:4	0	1	0	0	1	1	3
1:8	0	0	0	1	2	2	5
1:16	1	1	0	0	3	2	7
1:32	2	2	0	1	5	3	13
1:64	1	2	0	3	2	2	10
1:128	4	2	0	4	2	2	14
1:256	3	0	0	0	1	0	4
Anticomple- mentary	3	0	3	0	0	0	6
Total	24	12	12	14	20	18	100
No. of reactors	12	9	1	9	17	13	61
% reactors	50	75	8.3	64	85	72	61
No. tested		12	12	14			36
Virus recovered	*	1	1	Incomplete	*	*	2

*Not attempted.

⁹ Machiavello, A., *Virus and Rickettsial Diseases*, Harvard Univ. Press, Cambridge, Mass., 1941, p. 896.

But the sera of pigeons with high titers of complement-fixing antibodies to M P virus failed to react with antigen prepared with the virus of lymphogranuloma venereum. These observations indicate that factors other than antigenic constitution play a rôle in determining the cross reactions obtained in serological tests. They suggest, furthermore, that the use of pigeon serum may be of value in the differentiation of this group of infectious agents.

In addition, attempts were made to isolate virus from 36 individual pigeons irrespective of the serological status. Virus was recovered from the liver and spleen of one pigeon, and from the ovaries and kidneys of another (Table I). The virus appears in all respects to be typical of the virus of ornithosis in pigeons (Meyer). The fact that material from 34 additional birds studied simultaneously yielded no virus appears to eliminate the possibility that the virus was resident in the stock of mice as reported by Nigg.¹⁰ Control passages have likewise been negative.

(b) *Other Domestic and Wild Fowl.* Sera were obtained from 100 domestic turkeys and 45 chickens in different parts of the state and from 24 domestic ducks at the Detroit market. Sera from 55 wild ducks, 25 ringneck pheasants and 10 Hungarian partridges were also obtained (Table II). With 22 of the turkey sera fixation was observed in dilutions ranging from 1:4 to 1:16. Of the chicken

TABLE II.
Complement-Fixation Test with Serum from Domestic and Wild Fowl in Michigan.

Titer	Domestic			Wild		
	Chickens	Turkeys	Ducks	Hungarian Partridges	Pheasants	Ducks
0	36	67	15	10	25	55
1:2	2+ 4+ 3++++	1++++ 3+	1+++++	0	0	0
1:4	0	8++++ 2++++ 2+++ 4+	1+++++	0	0	0
1:8	0	1++++ 2+++	0	0	0	0
1:16	0	2++++ 1++	3+++++	0	0	0
Anticomplementary	0	16	4	0	0	0
Total	45	109	24	10	25	55

¹⁰ Nigg, C., *Science*, 1942, **95**, 49.

sera tested, 9 gave a reaction in the lowest dilution. With 5 of the sera of domestic ducks, reactions in serum dilutions of 1:2 to 1:16 were obtained. Tests with the sera of the wild fowl were completely negative. No attempts to recover virus were made. The results suggest that infection with ornithosis occurs in domestic turkeys and ducks while game birds, at least in Michigan, are relatively free of the infection.

Summary. Complement-fixation tests with the virus of meningo-pneumonitis in Michigan revealed that 61 of 100 pigeons reacted with the antigen; 41 of them in dilutions of 1:32 or greater. Pigeon serum fails to give the cross-reaction with lymphogranuloma venereum antigen which is commonly seen with human serum.

Reactions of variable intensity were obtained with 22 of 109 sera from domestic turkeys, with 5 of 24 sera from domestic ducks and, in the lowest dilution only, with 9 of 45 chicken sera.

No reactions were observed with the sera of 90 wild fowl.

The authors wish to express their appreciation for aid in obtaining blood samples from the various sources to Dr. H. J. Stafseth, Michigan State College; Dr. Stanley Whitlock, Michigan Department of Conservation; Mr. W. F. Van Dien, Director of Michigan State Game Farm; and Dr. J. G. Molner, Deputy Commissioner of Health, Detroit, Michigan. The psittacosis antigen was kindly furnished by Dr. K. F. Meyer of the Hooper Foundation, San Francisco; the lymphogranuloma antigen by Dr. Clara Nigg of the Squibb Institute, New Brunswick, New Jersey, to whom we express our thanks.

13777

Sinus Gland Extirpation in the Crayfish Without Eyestalk Removal.

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In view of the increasing volume of literature pertaining to endocrine activities of the crustacean sinus gland, it is unfortunate that the only simple and sure method of removal of these glands from the body has involved the removal of the total eyestalk. The eyestalk constituting the most important light receptor of the organism, contains, in addition to the sinus gland, several important nerve ganglia, the x-organ, and other possible endocrine sources. For this reason the usual removal of so much additional tissue with the glands leaves

the interpretation of the results of eyestalk removal somewhat uncertain, to say the least. Furthermore, such a function as control of retinal pigment migration, which is believed by a number of endocrinologists to be normally under the control of the sinus gland, has not yet been confirmed by such a crucial experiment as gland extirpation since this latter has also always involved removal of the effector mechanism as well. Nearly every recent investigator in crustacean endocrinology has at one time or another attempted to remove the sinus gland while leaving the rest of the stalk intact. The only published attempt to date¹ dealt with the highly transparent shrimp, *Palaeomonetes*. The technic developed in this case was not sufficiently satisfactory to be used as a routine laboratory method, and furthermore could not be used successfully upon the great majority of crustaceans whose exoskeletons were relatively opaque.

The simple technic described below has been devised to extirpate the sinus glands from crayfishes without eyestalk removal, and, in fact, with little apparent disturbance to the remaining functions of the stalk including vision. The operation can be performed rapidly, and after a little practice, bilateral extirpation of the glands in an animal can be accomplished in less than 5 minutes. With such speed, large numbers of animals may be operated in a relatively short time.

The method is as follows: The animal from which the glands are to be removed is first secured to the stage of a low magnification dissection microscope by means of rubber bands in order to leave both hands of the operator free for action. The animal is readily orientated beneath the rubber bands into nearly any desired position. The operator then holds a capillary glass aspirating tube in his left hand and a pair of 3C watch-maker's forceps in his right. With one point of the forceps, the eye is punctured approximately at point A (see Fig. 1) in the relatively thin cornea and the forceps point forced completely down through the eye. The eye is then carefully torn along the line A-B. The cornea of the eye at C and the visual elements of that region are carefully drawn away with the forceps and aspirator tube. The blood and diffusing pigment which immediately flow into the wound are also drawn out. As the fluids are aspirated, a concavity (D) is produced, and the sinus gland (E) comes into view at once, or it may easily be brought into view by teasing the region gently. The gland is then readily removed in one piece by means of the forceps. Since the gland is of a charac-

¹ Brown, F. A., Jr., Ederstrom, H. E., and Seudamore, H. H., *Anat. Rec.*, 1939, **75**, Suppl., 129.

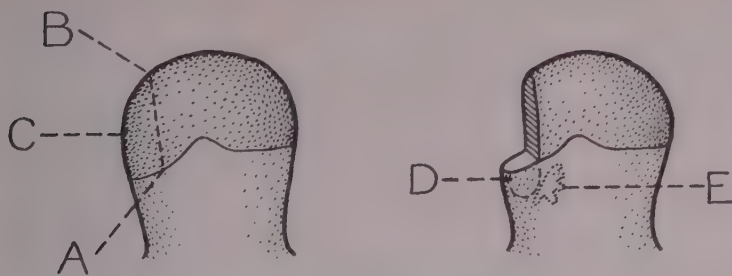


FIG. 1.

Dorsal view of the left eyestalk of *Cambarus immunis* showing incisions made for sinus gland removal and the approximate location of the gland.

teristic bluish-white tint and has a texture quite different from the surrounding tissue of the stalk it may be thoroughly removed even though the gland breaks into several pieces due to clumsy initial treatment.

This method of sinus gland removal gives a far lower percentage of operative deaths than does eyestalk extirpation. In several series, each involving sinus gland removals from 10 animals, I have had 100% survival from the operation itself. Animals from which the sinus glands have been removed in this manner behave outwardly much more normally than do animals without eyestalks. Their posture and responsiveness appear quite normal, and even vision appears not to be radically disturbed. The wound heals quickly, even though no precaution is taken to prevent bleeding.

These operated animals, judging from the brief experiments which have been performed to date, appear to show the lowered vitality and increased molting rate which certain published data^{2, 3} indicated should result from the removal of only the sinus glands.

² Brown, F. A., Jr., and Cunningham, O., *Biol. Bull.*, 1939, **77**, 104.

³ Kyer, Donald L., *Biol. Bull.*, 1942, **82**, 68.

Effect of Certain Hormones and Drugs on the Perfused Mammary Gland.*

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Although Ott and Scott¹ demonstrated that an extract of the posterior hypophysis would cause an ejection of milk from the alveoli of the mammary gland this phenomenon was not associated with the natural let down of milk until the work of Ely and Petersen,² who demonstrated that both the oxytocic and vasopressor substances of the posterior pituitary would cause an ejection of milk in the cow. Turner and Cooper³ reported the same for the rabbit. Ely and Petersen observed fright or the injection of epinephrin inhibited the natural response to the milking stimulus. Petersen and Ludwick⁴ showed that the substance responsible for the ejection of milk is humoral in nature and that the blood from excited cows has great vasoconstricting properties in perfused bovine mammary glands.

As a result of these observations, it was deemed advisable to test the effects of a number of substances upon the blood flow and the milk ejection mechanism in the bovine mammary gland. This report deals with 9 substances: oxytocin, pitressin, epinephrin, histamine, atropine, ergonovine, acetylcholine, Mecholyl (acetyl- β -methylcholine) and Lentin (carbamylcholine).

The experiments here reported were on bovine mammary glands perfused according to the technic of Petersen, Shaw and Visscher.⁵ The glands were obtained from a packing plant. The teats were cannulated to permit drainage of the milk immediately after the perfusion started and the cannula left *in situ* during the experiment. The blood pressure was maintained at 110 mg Hg pressure at all times. The substances tested were injected into the arterial blood as it entered the gland.

The number of experiments, dosage of the substance, effect on the

* Scientific Journal Series Paper No. 2009, Minnesota Agricultural Experiment Station. Prepared with the assistance of Works Projects Administration, Official Project No. 165-1-71-124.

¹ Ott, I., and Scott, J. C., *Proc. Soc. Exp. Biol. and Med.*, 1910, **8**, 48.

² Turner, C. W., and Cooper, W. D., *Endocrinology*, 1941, **29**, 320.

³ Ely, F., and Petersen, W. E., *J. Dairy Sci.*, 1941, **24**, 211.

⁴ Petersen, W. E., and Ludwick, T. M., *Federation Proc.*, 1942, **1**, 66.

⁵ Petersen, W. E., Shaw, J. C., and Visscher, M. B., *J. Dairy Sci.*, 1941, **24**, 139.

blood flow and the effect on the ejection of milk are given in Table I for each substance tested. As the rate of the blood flow and the amount of milk in the glands varied greatly in the different experiments, it is not advisable to quantitate the data on such a basis. That ten units of Pitocin would quantitatively express the milk from a gland has been ascertained in many experiments. Pitocin injections following the use of any other substance was, therefore, resorted to as a test for the completeness of the milk ejection.

Only Pitocin and acetylcholine caused complete evacuation of milk from the gland. Of these acetylcholine did not affect the rate of blood flow while Pitocin caused a decrease. The vasoconstricting effect of Pitocin may be attributed to the pitressor principle which it contains. Pitressin (10 units) caused a marked decrease in blood flow and an incomplete evacuation of milk as in all experiments varying quantities of milk were obtained with Pitocin following pitressin.

The mammary gland seems to be unusually sensitive to epinephrin, for as little as 0.05 mg caused a 50% reduction and 0.1 and 0.2 mg completely stopped the blood flow. In all cases some milk was ejected with this hormone. Histamine also proved to be a powerful vasoconstrictor in the mammary gland and produced milk ejection of approximately the same magnitude as epinephrin.

Ergonovine in 0.2 to 0.6 mg doses proved to have no effect upon the ejection of milk but caused some vasoconstriction.

While acetylcholine caused a complete ejection of the milk acetyl- β -methylcholine (Mechoyl) in as large and larger doses caused but a partial evacuation of milk from the gland. Carbamylcholine in

TABLE I.
Effect of 9 Hormones and Drugs upon the Blood Flow and Ejection of Milk in the Perfused Mammary Gland.

Substance	No. of exper.	Amt used	Effect on blood flow	Effect on milk ejection
Pitocin	12	10 units	decrease 8-20%	complete
Pitressin	6	10 "	" 40-60%	partial
Epinephrin	10	0.05-0.2 mg	" 50-100%	partial
Histamine	10	0.5-10 mg	" 50-100%	partial
Ergonovine	6	0.2-0.6 mg	" 10-20%	no effect
Acetylcholine	5	4.0-100 mg	no effect	complete
Acetyl- β -methylcholine	4	25.0-150 mg	" "	partial
Carbamylcholine	4	2-8 mg	slight increase	no effect
Atropine	8	2-4 mg	" "	" "
Acetylcholine following atropine	3	100 mg	no effect	" "
Acetyl- β -methylcholine following atropine	2	250 mg	" "	" "

2 to 8 mg doses had no effect upon milk ejection. Atropine tended to increase blood flow slightly and completely prevented any response to acetylcholine and acetyl- β -methylcholine but had no effect upon the action of Pitocin or pitressin.

From these observations it would appear that the mammary gland is innervated with the parasympathetics as well as the sympathetics.

The acetylcholine, Mecholyl and Pitocin used in these experiments were furnished by Merck and Company through the courtesy of Doctor D. F. Green.

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Studies on the Toxicity of Protamine.

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The parenteral administration of protamine has been suggested by Jorpes¹ and Ravdin² as a clinical method of terminating the action of administered heparin. Since the toxic effects of the protamines have not been extensively studied, further investigations seemed desirable.

Thompson³ and later workers have shown that relatively large doses of protamines produce dyspnea, a marked fall in the blood pressure, diminished blood coagulability, and in sufficient dosage, death. These symptoms were observed in several animal species. Thompson felt that the action of protamines could best be explained on the basis of a general toxic effect on the cardiovascular and respiratory systems. Postmortem studies on rabbits and mice, killed by subcutaneous injection of protamine, have been performed by Vartiainen and Marble.⁴ Hemorrhages in the thymus, lungs, and kidneys were the most constant findings.

Although Kern and Langner⁵ and earlier workers⁶ had failed to produce anaphylaxis with protamine, Walther and Ammon⁷ recently claimed to have produced fatal anaphylactic shock in guinea pigs

¹ Jorpes, E., Edman, P., and Thaning, T., *Lancet*, 1939, **237**, 975.

² Ravdin, I. S., *Am. J. Med. Sc.*, 1941, **201**, 299.

³ Thompson, W. H., *Trans. Roy. Acad. Med. in Ireland*, 1900, **18**, 426.

⁴ Vartiainen, I., and Marble, A., *J. Lab. and Clin. Med.*, 1941, **26**, 1416.

⁵ Kern, R. A., Langner, P. H., *J. A. M. A.*, 1939, **113**, 198.

⁶ Wells, H. G., *Physiol. Rev.*, 1921, **1**, 44.

⁷ Walther, G., and Ammon, R., *Klin. Wschr.*, 1939, **18**, 288.

with this substance. Their work, however, does not rule out the possibility that their animals died from direct toxicity of the shock dose (anaphylactoid shock) rather than from anaphylactic shock. Accordingly, we have investigated the effect in previously untreated animals of the shock dose employed by Walther and Ammon (120 mg/kg).

Method and Results. One to 3% aqueous solutions of salmine sulfate* were employed. Injections were made into the heart, saphenous vein, and peritoneal cavity of 23 guinea pigs and 3 rats. The results are summarized in Table I. The animals showed typical anaphylactoid symptoms,⁶ including weakness, dyspnea, and convulsions, leading to death. The heart beat continued after respiration had ceased. Hence it is probable that Walther and Ammon did not produce anaphylactic shock since their only proof rests on the

TABLE I.
Effect of Saline Sulfate on the Normal Animal.

Animal	Dose, mg/100 g body wt	Survival period (min.)
A. Intracardiac Injection.		
Guinea Pig	12	3.0
" "	12	3.0
" "	12	3.5
" "	12	2.0
" "	12	2.0
" "	12	5.0
" "	12	2.0
" "	12	3.0
" "	10	3.0
" "	10	2.5
" "	10	3.5
" "	6	18.0
" "	5	Survival
" "	3	" "
Rat	36	0.2
" "	12	3.5
" "	12	3.0
B. Intravenous Injection.		
Guinea Pig	12	2.0
" "	12	3.5
" "	12	2.5
" "	12	4.0
" "	12	3.5
C. Intraperitoneal Injection.		
Guinea Pig	12	2 hr 15 min
" "	12	1 " 55 "
" "	12	2 " 20 "
" "	12	17 "

* Kindly placed at our disposal by Eli Lilly and Company, Indianapolis, Indiana.

symptomatology which we have duplicated entirely in the normal non-sensitized animal.

In an attempt to explain these anaphylactoid symptoms, further experiments were conducted. It was found that the addition of salmine to the whole blood of human beings, dogs, rabbits, cats, guinea pigs, rats, and mice produced a thready precipitate. In the human, dog, cat, and rat blood, gross agglutination of the blood corpuscles occurred. However, in rabbit, guinea pig, and mouse blood this phenomenon did not take place. Upon the addition of salmine to plasma a heavy white flocculent precipitate appeared. Serum, when treated similarly, exhibited only turbidity. These effects occurred in the whole blood, plasma, and serum in both the presence and absence of such anticoagulants as heparin, citrate and oxalate.

Ten mg of salmine per ml of plasma were required to produce maximum precipitation. Analysis of the total protein before and after such treatment with salmine showed removal of a quantity somewhat larger than the determined fibrinogen content. It was found that the precipitate could be redissolved in Ringer's solution or plasma by the addition of polyvalent ion salts or by a change in pH. After treatment of plasma with 10 mg of salmine per ml, 25% saturation with $(\text{NH}_4)_2\text{SO}_4$ yielded no precipitate. Upon increasing the concentration to 50% and 100%, however, voluminous precipitates resulted.

Since the addition of salmine to blood produced a precipitate, and

TABLE II.
Effects of Salmine Sulfate upon Perfusion Flow through Organs of Guinea Pig.

Organ perfused	Perfusion fluid	Injection dose (mg)	Control rate of flow (ml/min)	Min. rate of flow (ml/min)	Interval between inj. and min. flow (min)
I. Lung	Ringer's Solution	60	20.0	20.0	
"	Dog whole blood				
"	(oxalated)	60	10.4	0	2.5
"	"	10	12.0	0	4.0
"	"	20	10.0	0	7.0
"	"	20	15.0	0	3.0
"	"	20	9.0	0	0.25
"	"	30	8.0	0	0.5
"	"	20	6.4	0	1.0
II. Liver	Dog Serum	20	19.2	18.2	
"	"	40	20.0	20.0	
"	Guinea pig whole blood (citrate)	20	8.2	0.6	3.0
"	"	20	7.2	0	8.0

in some animals cell agglutination, it seemed possible that these changes would have vascular occlusive effects. To test this hypothesis, salmine was added to blood flowing into perfused organs. The results are presented in Table II. Dog blood was used in most of these experiments because it was difficult to secure a sufficient quantity of guinea pig blood. It is seen that the addition of 10 mg or more of salmine to either dog or guinea pig whole blood resulted in rapid decline or cessation of blood flow. On the other hand no such effect was obtained upon the addition of salmine to dog serum or Ringer's solution.

Discussion. The data in Table I demonstrate that the dose of salmine which Walther and Ammon interpreted as causing death by anaphylaxis actually produced death in previously untreated guinea pigs. The *in vitro* experiments conducted on the blood suggest a more reasonable explanation of the toxicity of protamines than those proposed by previous authors.^{8, 4, 7} The data indicate that salmine exerts a toxic effect by the formation of multiple emboli. The precipitate noted when salmine was added to whole blood, as well as the agglutination of cells observed in some species, probably caused the vascular occlusive effects found in the perfusion experiments.

The studies of the salmine precipitate lead to the view that, in suitable concentrations, salmine will precipitate certain blood proteins, essentially fibrinogen.[†] By virtue of its high isoelectric point, salmine at blood pH has a positive charge while the plasma proteins are negatively charged. Hence, it is likely that salmine combines with these oppositely charged colloids. The peptizing effect of pH changes and of the addition of polyvalent ion salts on the salmine precipitate lend further support to this view. Abramson⁸ has pointed out that "mutual precipitation of charged colloids is observed only when at least one of the substances is easily precipitated by electrolytes." On this basis it is not surprising that fibrinogen, which is most easily salted out, is precipitated by salmine.

Of equal importance is the effect of salmine upon the erythrocytes. It is probably not a coincidence that the animal species shown by Jaques⁹ and Jorpes¹ to be most sensitive to protamine are also those whose red blood cells are agglutinated by this agent. On the con-

[†] Since the writing of this report Mylon, Winternitz, and de Suto-Nagy have published a method for determination of fibrinogen with protamine (*J. Biol. Chem.*, 1942, **143**, 21).

⁸ Abramson, H. A., Moore, D. H., *J. Lab. Clin. Med.*, 1940, **26**, 174.

⁹ Jaques, L. B., Charles, A. F., and Best, C. H., *Acta Med. Scand.*, 1938, Supp., **90**, 190.

trary, the species which are more resistant to protamine show absence of red cell agglutination by this agent.

On the basis of these findings, the intravenous use of large doses of protamine in clinical practice would seem not to be without danger.

Summary. 1. Intravenous administration of the protamine, salmine sulfate, to untreated guinea pigs and rats produced death at dose levels of 6-12 mg/100 g. Intraperitoneal injection delayed these effects. 2. The addition of salmine sulfate to whole blood or plasma resulted in the formation of a flocculent protein precipitate. Addition to whole blood caused hemagglutination in the human, dog, cat, and rat. 3. The addition of salmine sulfate to whole blood perfusing isolated organs caused cessation of flow. Addition of comparable amounts to Ringer's fluid and serum perfusing organs was without effect upon the rate of flow. 4. These experiments suggest the possibility that protamines exert toxic effects through embolic vascular phenomena.

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Studies on Peptic Inhibition.

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This report is a study of peptic activity as affected by the various substances used clinically in the treatment of gastro-duodenal ulcerative disease as well as of pepsin inactivators. Our specific objective was to learn whether these substances act directly on the pepsin or indirectly through the medium of a pH change.

Hammarsten¹ and Helmar, *et al.*,² showed that peptic activity decreases as the pH increases from 1 to 7, being completely inactivated at pH 7. Recently Komarov³ and Schiffrin⁴ reported that various aluminum and magnesium compounds inhibit peptic activity at the optimum pH range, from 1 to 2. Their use of Mett tubes to determine peptic activity plus their procedure of centrifuging

¹ Hammarsten, O., *Hoppe Seyler's Z.*, 1922, **121**, 261.

² Helmer, O. M., Fonts, P. J., and Zervas, L. G., *Am. J. Dig. Dis.*, 1934, **1**, 120.

³ Komarov, S. A., and Komarov, O., *Am. J. Dig. Dis.*, 1940, **7**, 166.

⁴ Schiffrin, M. J., and Komarov, S. A., *Am. J. Dig. Dis.*, 1941, **8**, 215.

out the undissolved material made this method unsatisfactory for our use. The Mett tube method possesses the disadvantage of a slow rate of digestion as well as the difficulty in obtaining a reproducible substrate. Centrifugation removes the adsorbed pepsin but does not prove its inactivation.

Methods. We found the Anson-Mirsky^{5, 6} hemoglobin method as modified by Beazell⁷ reliable and accurate. The source of pepsin was either a solution of granular pepsin* in N/10 HCl or gastric juice collected from a Pavlov pouch dog.

Our procedure was as follows: In one-half of our experiments the substances studied were incubated with gastric juice for 15 minutes at 37°C, determining the pH before and after incubation. Then an aliquot portion of the incubated material was added to 5 cc of a 4% hemoglobin substrate. This was incubated for 15 minutes at 37°C and the undigested hemoglobin precipitated with 10 cc of 4% trichloroacetic acid. The amount of digestion was determined by measuring the tyrosine, colorimetrically by Folin's method, in an aliquot portion of the filtrate.

In the second group of experiments exactly the same procedure was followed except that before incubation the substances used were brought to a pH between 1 and 2 with HCl. The substances tested were aluminum hydroxide, aluminum phosphate, magnesium trisilicate, and Mucin.

In addition to these therapeutically used substances, Dr. H. B. Bull of the Department of Physiological Chemistry, suggested using a protein denaturant.⁸ This denaturant was a commercial sample of sodium lauryl sulfate.^{9, 10, 11} It is a surface active agent which has the property of denaturing proteins in very small concentrations.

Results. Unbuffered aluminum hydroxide, aluminum phosphate, magnesium trisilicate, and Mucin inhibited peptic activity. From Graphs I, III, V, and VII it can be seen (curves A) that as the concentration increased the peptic activity decreased. However, these same graphs show that as the concentration increased the pH increased (curves B), the two curves paralleling each other. The one exception was sodium lauryl sulfate where the pH remained inde-

⁵ Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1932, **16**, 59.

⁶ Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1938, **22**, 79.

⁷ Beazell, J. M., Schmidt, C. R., Ivy, A. C., and Monaghan, J. G., *Am. J. Dig. Dis.*, 1938, **5**, 661.

* 1/10,000 spongy pepsin (3 × U.S.P.) Generously supplied by Armour & Co.

⁸ Bull, H. B., and Neurath, H., *J. Biol. Chem.*, 1937, **118**, 163.

⁹ *J. Ind. and Eng. Chem.*, 1941, **33**, 17.

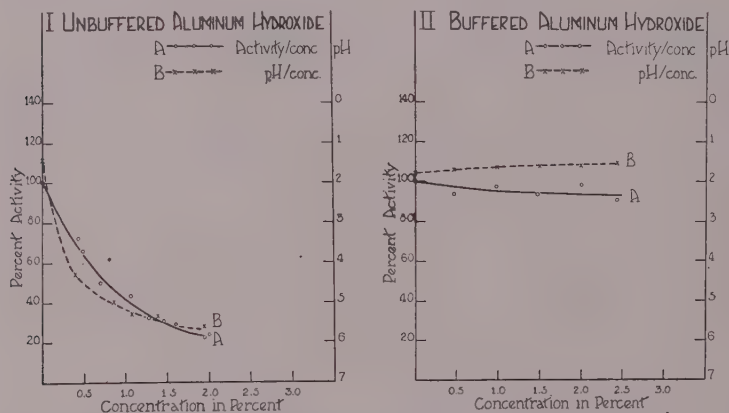
¹⁰ Degering, *J. Chem. Educ.*, 1941, **18**, 102.

¹¹ Blair, C. M., *J. Chem. Educ.*, 1941, **18**, 246.

pendent of the concentration (Graph IX), but peptic activity was markedly inhibited with as small a concentration as 0.2%.

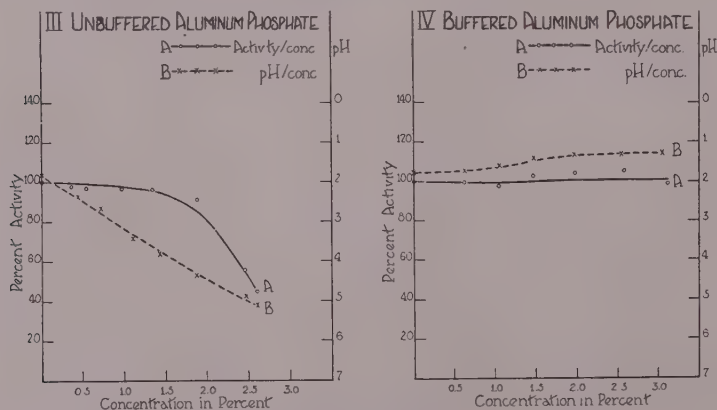
In the other half of the experiments where the pH was kept constant by buffering with HCl (Graphs II, IV, VI, VIII, curves B) the peptic activity (curves A) remained practically unchanged.

Discussion. With the exception of sodium lauryl sulfate peptic inhibition occurred only when the concentration was sufficient to markedly alter the pH, as is shown by the absence of inhibition when the pH is kept constant. With sodium lauryl sulfate, however, we found that pepsin inactivation occurred with no pH change.



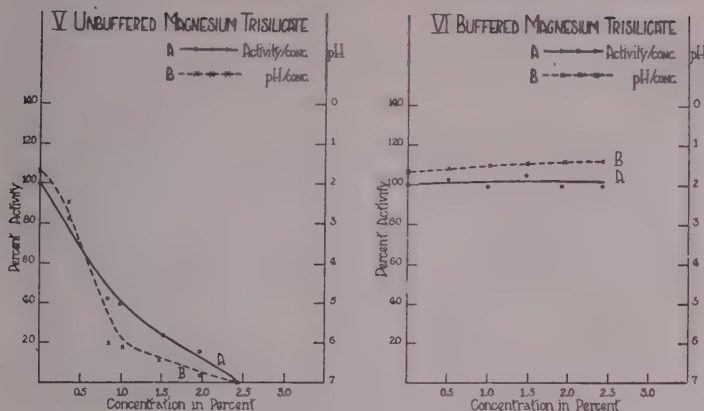
GRAPHS I AND II.

Increasing concentration of aluminum hydroxide decreases peptic activity only when the aluminum hydroxide is unbuffered.



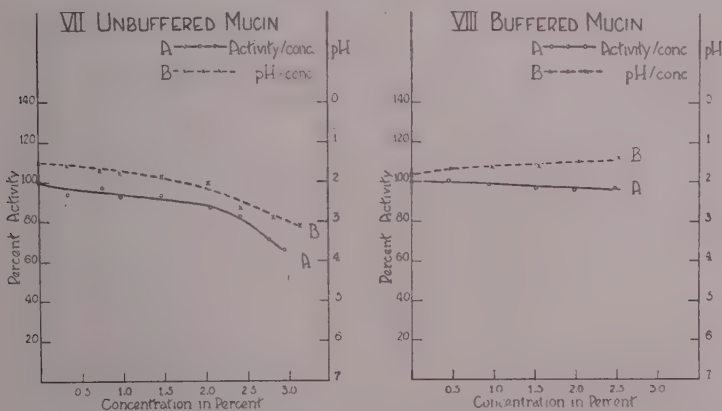
GRAPHS III AND IV.

Increasing concentration of aluminum phosphate decreases peptic activity only when the aluminum phosphate is unbuffered.



GRAPHS V AND VI.

Increasing concentration of magnesium trisilicate decreases peptic activity only when the magnesium trisilicate is unbuffered.



GRAPHS VII AND VIII.

Increasing concentration of gastric mucin decreases peptic activity only when the gastric mucin is unbuffered.

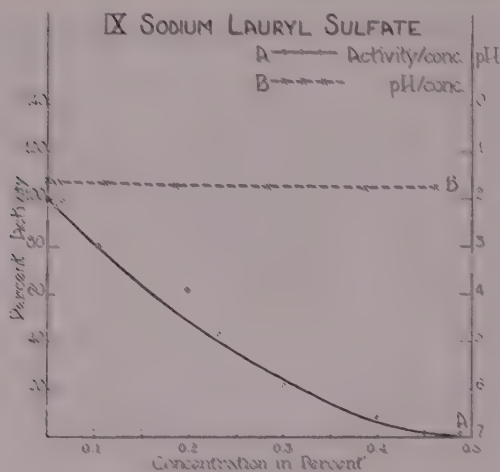
We have reported previously¹² that similar peptic inhibition by sodium lauryl sulfate occurs *in vivo* and will markedly increase the survival time of dogs with ulcers induced experimentally by the massive histamine injection method of Code, *et al.*^{13, 14}

Conclusions. 1. Aluminum hydroxide, aluminum phosphate, mag-

¹² Shoch, D., and Fogelson, S. J., *Quart. Bull. N. U. M. S.*, 1942, in press.

¹³ Walpole, S. H., Varco, R. L., Code, C. F., and Wangenstein, O. H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 619.

¹⁴ Varco, R. L., Code, C. F., Walpole, S. H., and Wangenstein, O. H., *Am. J. Physiol.*, 1941, **33**, 475.



GRAPH IX.

This shows how sodium lauryl sulfate decreases peptic activity without any alteration in pH. Note effectiveness of dilute concentrations.

nesium trisilicate, and mucin inhibited peptic activity by altering the pH, the percent of inhibition being directly related to change in pH. 2. Of these 4, magnesium trisilicate was the most effective inhibiting agent, exerting the greatest effect on the pH. 3. Of the substances used, sodium lauryl sulfate alone completely inhibited peptic activity without any alteration in pH.

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Forced Ovulation of Normal Ovarian Follicles in the Domestic Fowl.

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The induction of multiple ovulation following intravenous injection of a luteinizing preparation into hens pretreated with pregnant mares' serum administered subcutaneously for 0 to 100 days has been described.¹ The results set forth in this paper show that ovulation of normally maturing follicles can be forced by at least 17 hours. While the absolute prematurity of ovulation imposed on

¹ Fraps, R. M., and Riley, G. M., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 253.

the maturing follicle seems perhaps small in comparison with some results on mammalian species, the interval is of striking magnitude relative to the normal interval, 24 to 27 hours, between successive ovulations in laying hens. In the regularly laying hen the time of next expected ovulation as well as the time of induced ovulation² can be predicted with relatively high accuracy, contributing greatly to interpretation of results based on ovulation of follicles by even a few hours prematurity.

Time Relations in Normal and Induced Ovulation. Estimation of prematurity of ovulation following experimental treatment was made as follows:

(1) The time of next expected normal oviposition was estimated on the basis of the hen's previous record of lay. One half-hour beyond this time was taken as the time, to within one hour, of next expected normal ovulation.^{3, 4, 5}

(2) At autopsy of hens, generally 8 to 11 hours following injection, the position of the ovulated yolk in the oviduct was determined by inspection, and by reference to Warren and Scott's³ time schedule of progression of the yolk through the oviduct the time of actually induced ovulation was estimated. When ovulated yolks were found in the body cavities of autopsied hens, the time of ovulation was taken as the average time at which ovulation occurred in those hens of the same or of a comparable group with yolks in their oviducts. In general, estimates of time of induced ovulation should be accurate to within ± 0.5 hour.

Having established the time of expected normal ovulation and the time of induced ovulation, prematurity of ovulation was obtained as the difference, expressed in hours, between the actual and the normally expected events. All calculations were made for each hen individually.

Materials and Methods. White Leghorn and Rhode Island hens in their first or second year of production were used in all experiments. Birds of the 2 breeds and ages were so distributed to various experimental groups that reaction differentials arising from these sources were minimized. All hens were kept in individual laying cages under a constant 14-hour light-day for at least 2 weeks before being subjected to experimental treatment. During this period—in

² Fraps, R. M., Riley, G. M., and Olsen, M. W., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 513.

³ Warren, D. C., and Scott, H. M., *Poultry Science*, 1935, **14**, 195.

⁴ McNally, E. H., and Byerly, T. C., *Poultry Science*, 1936, **15**, 280.

⁵ Phillips, R. E., and Warren, D. C., *J. Exp. Zool.*, 1937, **76**, 117.

most instances for a considerably longer time—hourly laying records were maintained from 8:00 a.m. to 4:00 p.m. to provide the data required for estimation of prematurity of induced ovulation.

Four hormone preparations were injected intravenously with effective result. The designations and assay data of these preparations follow:

(1) *LH Preparation*.^{*} A luteinizing preparation derived from horse anterior pituitaries, assaying 20 rat seminal vesicle units (Fevold test) per cc, and containing also follicle stimulating hormone in considerable quantities. This is the same preparation previously used in bringing about multiple ovulations in pretreated hens.¹

(2) *Prephysin* (Chappel Prephysin-veterinary).[†] Primarily the follicle stimulating principle, but containing also "a relatively small amount of the luteinizing hormone." Assayed in rat units for follicle stimulating hormone: 50 rat units per cc.

(3) *Gonadin* (Gonadin Serum veterinary).[‡] The sterile serum of pregnant mares, standardized to contain 50 rat units per cc.

(4) *Anteron*.[§] Concentrate of the active gonadotrophic principle of the serum of pregnant mares, standardized in international units, and used in the present experiments at ca. 1200 I.U. per cc diluent.

No attempt was made to reduce the gonadotrophic potencies of the four preparations to a single standard, since this was deemed secondary to the demonstration that each was effective in bringing about premature ovulation of the normally maturing ovarian follicle.

Results. The data presented in Table I show that the rupture of ovarian follicles can be induced by at least 17 hours before normally expected ovulation (Anteron, 1200 units, 10:00 a.m. injections). This is not necessarily the maximum prematurity of follicles in which ovulation can be induced. In one hen injected with LH-Chappel at the 20 unit level two follicles were caused to rupture. One of these was presumably of ca. 27, the other of ca. 4.0 hours prematurity. The weights of the two ovulated yolks were 19.4 and 16.9 g, the order of difference in weights being that expected in ovarian follicles due to ovulate on successive days. On the other hand, the injection of Anteron at the 1200 unit level, a level of sub-maximal effectiveness in causing ovulation of follicles of 11 hours or more prematurity, did not bring about ovulation of more than a single follicle in any hen injected for effect at almost the same time

^{*} Extract especially prepared by Chappel Laboratories, Rockford, Ill.

[†] Chappell Laboratories, Rockford, Ill.

[‡] Cutter Laboratories, Chicago, Ill.

[§] Supplied by courtesy of Schering Corporation, Bloomfield, N.J.

TABLE I.
Ovulation of Single Follicle Following Intravenous Injection of Hormone Preparations into Hens.

Preparation	Quantity, units	Time of injection	Hens			Estimated prematurity	
			Injected, No.	Ovulating, No.	%	Avg hrs	Range hrs
LH (Chappel)	20	11:45 p.m.	7	7*	100	3.48	2.3- 4.1
	20	7:30	3	3	100	8.40	8.3- 8.5
	20	4:00	3	3	100	11.20	10.8-11.7
	8	4:00	4	4	100	10.30	10.8-11.7
	4	4:00	3	3	100	10.30	9.7-10.8
	2	4:00	3†	0†	—	ca.10.0†	—
Prephysin (Chappel)	100	3- 4:00	3	3	100	11.8	11.2-12.1
	50	3- 4:00	3	2	67	11.0	11.0-11.1
	50	9:00	3	2	67	6.3	5.7- 7.0
Gonadin Serum (Cutter)	150	4- 5:00	10	7	70	10.0	8.8-11.0
	100	4:00	3	2	67	11.8	11.1-13.1
	50	3- 4:00	6	2	33	10.6	9.5-12.7
	50	12:00 midnight	3	2	67	3.5	2.4- 5.6
	40	8:00 p.m.	3	1	33	4.9	4.0- 6.3
	20	8:00	3	0	—	ca.5.0†	—
	10	8:00	3	0	—	ca.6.0†	—
Anteron (Schering)	1200	10:00 a.m.	12	3	25	16.94	14.2-17.8
	1200	12:00 noon	12	7	58	14.89	14.1-15.7
	1200	4:00 p.m.	8	7	88	11.14	9.0-13.2
	1200	3:00 a.m.	11	11	100	-0.64	-1.4- 2.9
	400	4:00 p.m.	4	1	25	10.88	10.2-11.6
	120	4:00	4	0	—	ca.10.50†	—

*Two follicles ovulated in one hen of this group.

†No follicle was ovulable in one hen of this group.

‡These figures represent the approximate time at which ovulation might have been expected at next highest effective injection level.

that normal ovulation was expected (Table I, Anteron, -0.64 hour prematurity).

It should be noted that the negative value of estimated prematurity in this group of hens indicates that in some birds ovulation may have occurred independently of and prior to effect by the injected hormone; the fact that the ovulated yolk was found in almost precisely the same portion of the oviduct in every hen, each autopsied exactly 10 hours after injection, is however sufficient proof of induced ovulation in those hens normally due to ovulate later than was actually recorded.

The effectiveness of injection at submaximal levels is dependent to a high degree upon the maturity of the ovulating follicle. The results obtained following injection of Anteron at the 1200 I.U. level and at differing times before expected normal ovulation (Table I) bring out the relation clearly. A similar relation appears following injection of Gonadin Serum, 50 units inducing ovulation in only 33%

of hens with follicles of 10.6 hours prematurity, but in 67% of hens with follicles of 3.5 hours prematurity.

Comment. The results on forced rupture of the ovarian follicle of the domestic hen under the procedures reported in this paper indicate that consummation of the ovulatory process requires the action of a specific hormone or combination of hormones upon relatively mature follicles. The nature of the effective hormone or combination of hormones will be considered elsewhere. Of more immediate interest is the demonstration, not unexpected, that the effectiveness of ovulatory preparations decreases with increasing prematurity of the ovarian follicle. The data indicate also that the "relative prematurity" of a follicle, as measured by ovulatory response, is in part a function of level of injection of the ovulatory preparation; there appears nevertheless to be a limit beyond which increasing dosages will have no effect. Follicles of not more than 8 or 10 hours prematurity are ovulable in a high degree under the conditions described in this paper. It seems probable, though certainly not proved, that normal ovulation in the fowl is consequent upon the sudden release of an appropriate hormone into the blood stream. This hypothesis is now under investigation.

Summary. Ovulation of the normally growing follicle of the hen's ovary can be induced by as much as 17 hours prior to time of expected normal ovulation by intravenous injection of appropriate hormones. A luteinizing preparation from horse anterior pituitaries effected ovulation of follicles 10 to 11 hours prior to time of normally expected ovulation in 100% of injected hens at a level of 4 rat units (Fevold rat seminal vesicle test). The commercial preparations Prephysin, Gonadin serum and Anteron were effective when administered at sufficiently high levels. At appropriate submaximal injection levels the percentage of ovulating hens decreased with increasing prematurity of the ovarian follicles; this effect is more pronounced in follicles of relatively great prematurity at the time of effect of the injected hormone.

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Time Required for Induction of Ovulation Following Intravenous Injection of Hormone Preparations in Fowl.

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In both the normal (non-pretreated)¹ and the pretreated² hen, ovulation is induced by the intravenous injection of appropriate hormone preparations, and it has become a matter of interest to determine the interval between injection of the ovulatory preparation and rupture of the ovarian follicle. In most hens under the conditions of experiment thus far employed the interval is between 6.5 and 8.5 hours, indicating a remarkably direct effect of the intravenous injection.

Materials and Methods. Only White Leghorn hens approximately one year of age were used in the tests of pretreated hens. White Leghorn and Single Comb Rhode Island Red hens of differing ages were injected in establishing the ovulatory interval for normally laying (non-pretreated) hens. Most of the hens in this latter group served simultaneously in supplying data on rupture of the premature normal follicle.¹ Additional hens were used in the present connection, particularly in checking the minimal time of response to the ovulatory injection.

All hens were kept in laying batteries under a constant 14-hour light day, and hourly laying records were maintained for two weeks prior to and during the experimental period.

The pretreated hens were all intravenously injected with the luteinizing preparation from horse anterior pituitaries previously used in the induction of multiple ovulation.² In the normally laying (non-pretreated) hens the same preparation was used in addition to the predominantly follicle-stimulating preparation, Prephysin, and two preparations from the serum of pregnant mares, Gonadin serum and Anteron.* Each preparation was administered over a considerable range of dosages.

The procedure used in pretreatment was essentially that described

¹ Fraps, R. M., Olsen, M. W., and Neher, B. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **50**, 308.

² Fraps, R. M., and Riley, G. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **49**, 253.

* These products are briefly described in citation 1, above.

earlier,² 100 rat units of pregnant mares' serum being administered subcutaneously, daily, through 8 days. After pretreatment the ovulatory injection (the luteinizing preparation only) was made at the uniform level of 20 rat units (Fevold seminal vesicle test) per hen. The injected hens were sacrificed at known times and upon autopsy the number of ovulated follicles was determined. If an ovulated yolk had entered the oviduct, the oviducal level at which it was found was ascertained as carefully as possible. The time required by the yolk, following ovulation, to reach definitive regions of the oviduct was then estimated on the basis of the time relations described by Warren and Scott.³ The time of ovulation was thus arrived at, and the difference between known time of injection and estimated time of ovulation was taken as the interval required for ovulatory effect. This estimate of the ovulatory interval applies of course only to the yolk picked up by the oviduct. In some hens ovulated yolks were found only in the body cavities at autopsy; it was assumed that in these hens ovulation had occurred not more than 15 minutes prior to the time of autopsy.³ The progress of ovulated yolks may not be entirely normal in pretreated hens, and the data reported for such hens are accordingly limited to groups in which yolks were in the anteriormost levels of the oviduct or free in the body cavities of autopsied hens.

Estimation of time of ovulation of yolks in normally laying hens (non-pretreated) was identical with procedures just described except that estimates were made also for yolks in more advanced oviducal regions in a number of instances, but in no case for yolks beyond the isthmus.

Results. (1) *Pretreated Hens.* A considerable number of pretreated hens were autopsied at various intervals following injection of the ovulatory preparation, intervals which proved much too long to permit of accurate estimates of times of ovulation and hence of the ovulatory interval. Results on these birds and others which served to determine the approximate ovulatory interval need not be discussed here.

Injection and autopsy data on 4 groups of hens sacrificed at around 7 hours following the ovulatory injection are given in Table I. Fourteen of the 15 injected hens had ovulated at the time of autopsy, the single failure occurring in a hen of the group injected at 8:30 a.m. The number of ovulations per ovulating hen also was lowest in the 8:30 a.m. group, the average being 1.5. While the differences between the 8:30 a.m. and the other groups may be

³ Warren, D. C., and Scott, H. M., *Poultry Sci.*, 1935, **14**, 195.

TABLE I.
Time of Ovulation Following Intravenous Injection of a Luteinizing Preparation,
20 Units per Hen, into White Leghorn Hens Pretreated with Pregnant Mares'
Serum, 100 Units Daily, through 8 Days.

No. of hens	Time of ovulatory injection	Injection to autopsy, hr	Ovulations per ovulating hen, No.	Injection to ovulation	
				Avg, hr	Range, hr
5	8:30 a.m.	7.2	1.5	7.0	6.6-7.2
4	1:00 p.m.	7.1	2.3	6.7	6.3-7.1
3	6:00	7.1	3.3	6.9	6.6-7.1
3	9:15	7.1	2.7	6.6	6.1-7.0

of some significance in other connections, there appears to be no consistent trend in either average time from injection to ovulation or in range of the ovulatory interval in hens injected at different times of the day.

The time from injection to ovulation, averaged by groups, varied by only 0.4 hour, and the range in ovulatory interval for all ovulating hens was from 6.1 to 7.2 hours. Since the average time from injection to autopsy was only 7.1 hours, the average time from injection to ovulation, 6.8 hours for the 14 ovulating hens, cannot possibly be more than 0.3 hour in error on the maximal side. The time of first ovulation following injection is not so positively determined, since this involves estimation of age of yolks in oviducts and must assume that the first yolk to be ovulated is also the first to be picked up by the oviduct. The minimal figures for the ovulatory interval (Table I, "range") are accordingly to be considered high rather than low.

(2) *Normally Laying (Non-pretreated) Hens.* The interval between injection of the ovulatory preparation and ovulation was estimated for each of 69[†] hens. The percentages of hens ovulating during one-half hour periods from 6.5 to 10.0 hours following the ovulatory injection, are shown in Fig. 1. Ninety percent of all ovulations occurred between 6.5 and 8.5 hours following the ovulatory injection, the largest percentage, 31.9, falling between 7.0 and 7.5 hours, and almost equal percentages, 21.7 and 23.3, occurring in the 6.5-7.0 and 7.5-8.0 hour periods respectively.

Seven yolks were found in body cavities of hens autopsied between 8.1 and 9.6 hours following ovulatory injection, and it is perhaps a question whether the oviduct failed to pick up these or whether the hens were killed very soon after ovulation had occurred. The latter

[†] The same hens were used in the induction of ovulation prematurely (citation 1, above); of the 70 prematurely ovulating hens one was omitted in the present connection because of inadequate autopsy record.

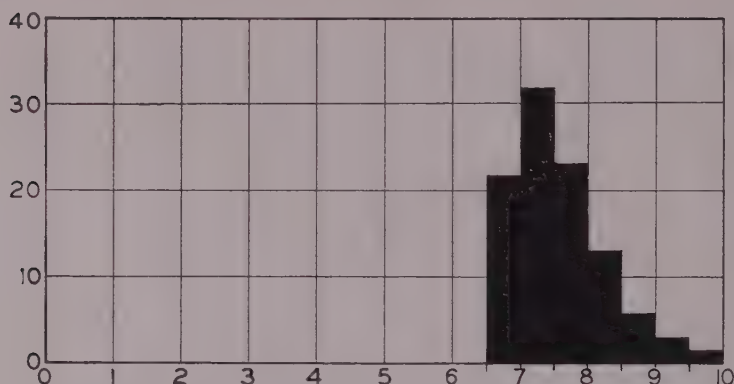


FIG. 1.

Distribution of induced ovulations, non-pretreated hens, in time from injection of ovulatory preparation. Abscissas, time in hours from injection (0 hour); ordinates, percentage of ovulations occurring within indicated 0.5-hour intervals.

possibility seems more probable, in some instances, at least, since yolks were found in infundibula of 2 hens, one killed during the 8.0-8.5 and the other during the 9.0-9.5 hour periods following injection. Furthermore, the mean estimated ovulatory interval decreases as yolks are found in increasingly caudal oviducal regions. The average interval for yolks found in the infundibulum is 8.8 hours, in the magnum, 7.6 hours, and in or very close to the isthmus, 6.9 hours. Such a relation is of course expected, since in general these yolks first to be ovulated would also be the first to enter the oviduct.

In order to check directly the minimal ovulatory interval 12 hens were divided into 3 groups of 4 each and sacrificed at precisely 6, 6.5, and 7.0 hours following injection of 1200 units Anteron per hen. No ovulations occurred in the hens killed at 6 hours, one occurred while the ovary of a hen in the 6.5 hour group was being removed, and 2 of the 4 hens killed at 7 hours had ovulated. This result is in good agreement with the relations shown in Fig. 1.

Comment. The time from injection of the ovulatory preparation to ovulation was, on the average, significantly shorter and the interval showed considerably less variation in pretreated hens than in non-pretreated hens. It should be emphasized, however, that in the pretreated hens—all of the same breed and age—a uniform, relatively high dosage of a potent ovulatory preparation was administered, whereas the non-pretreated hens were of differing breeds and ages into which injections of four different preparations were made at varying dosage levels to induce ovulation of follicles in varying

degrees of maturity. Furthermore, it may be assumed that most of the ovulating follicles of the pretreated ovary are, in consequence of pretreatment, wholly mature at the time of injection of the ovulatory preparation and therefore respond more uniformly to the ovulatory stimulus.

The minimal interval between injection and ovulation in either pretreated or non-pretreated hens appears to be appreciably less than are the intervals for even the most reactive of mammalian ovaries. However, the differences are not of large order, and the approximate similarity of interval rather than differences should perhaps be remarked upon. In any event, the response of the avian follicle within 6 to 9 hours is the more striking in view of the relatively enormous size of the ovarian follicle, the structural and physiological peculiarities associated with the deposition of deutoplasm, and the marked differences in ovulatory sequences of mammals in comparison with birds generally and with the domestic hen specifically.

Summary. The time at which ovulation occurs following intravenous injection of appropriate hormone preparations into pretreated and normally laying (non-pretreated) hens has been determined. The interval in pretreated hens averaging more than 2 ovulations per hen at time of autopsy was 6.8 hours, range 6.1-7.2 hours. Ninety percent of all normally laying hens (69) ovulated from 6.5 to 8.5 hours following ovulatory injection, the remaining 10% of injected hens requiring up to 9.6 hours for ovulation. The non-pretreated hens were injected with 4 different hormone preparations administered at differing dosage levels and at times calculated to effect ovulation of follicles at varying degrees of prematurity. The minimal ovulatory interval recorded for pretreated hens was 6.1 hours, for non-pretreated hens, 6.5 hours.

13783

Effect of Air Currents on Plasma Prothrombin.*

ARMAND J. QUICK.

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Tocantins¹ recently reported that plasma exposed to air currents

* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

¹ Tocantins, L. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **49**, 251.

showed a rapid diminution of prothrombin activity and that addition of CO_2 restored this loss. In view of the importance that these results have in the quantitative determination of prothrombin, and in the possible solution of the problem of preventing the spontaneous loss of this clotting factor in stored blood, it seemed important that these experiments be repeated.

Four cc of oxalated plasma were put in a pyrex test tube (25x100 mm) fitted with a 2-hole rubber stopper through which were inserted 2 glass tubes. One of the tubes nearly reached the surface of the plasma. The test tube was tilted to expose a maximum area of plasma surface, and a stream of air (first passed through a dilute solution of sodium hydroxide to remove CO_2) was blown over the plasma, kept at 38°C . The "prothrombin time" was determined by the author's method² and the CO_2 content by Van Slyke's original volumetric method. The loss of volume by evaporation was restored by adding distilled water.

The results indicate that the prothrombin of dog and rabbit plasma is not demonstrably altered by one hour aeration even though considerable CO_2 is lost. Human plasma aerated at room temperature likewise does not lose any significant amount of prothrombin activity in one hour. At 38°C , however, a definite loss of prothrombin occurs, which the addition of CO_2 does not restore. It appears, therefore, that the decrease of prothrombin is due to direct oxidative destruction or to the formation of an oxidation product in plasma which inactivates prothrombin, rather than to the loss of CO_2 from the plasma.

TABLE I.
Effect of Aerating Oxalated Plasma on Prothrombin Activity.

Type of plasma	Temp. $^\circ\text{C}$	Before aeration		After one hour aeration		Loss of vol., cc
		"Prothrom- bin time" sec.	CO_2 content vol., %	"Prothrom- bin time" sec.	CO_2 content vol., %	
Rabbit	38	6		6		2
Dog	38	6	58	6	38	1.5
Human	I 25	12		12*		0.7
	II 38	12		13½		1.4
	III 24	11½	57	11½	33	1.3
	IV 38	12	63	14	35	1.6
				14½	53†	

*An additional hour of aeration did not affect the prothrombin time.

†After saturating with CO_2 of alveolar air.

² Quick, A. J., *Am. J. Clin. Path.*, 1940, **10**, 222.

13784 P

Excretion Rate of Hippuric Acid in Man.

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The synthesis of hippuric acid as a test of liver function is valid only if the rate of excretion of this acid is greater than the speed of formation. It is desirable, therefore, to know how rapidly hippuric acid is excreted. Hirsheimer¹ found that when 2.26 g of sodium hippurate (equivalent to 2 g of hippuric acid) were injected intravenously, approximately 60% was excreted by normal pregnant women in 30 minutes. In patients suffering from various types of toxemias of pregnancy, the excretion was frequently diminished. Smith and Schwei² reported that when 1 g of hippuric acid was injected intravenously into a rabbit weighing 3 kg, 67% was excreted in 30 minutes; after injecting 2 g of the acid, 43.5% was eliminated, and after 3 g only 34.6% in the half-hour period.

In the present study, the procedure of Hirsheimer was followed except that the output of hippuric acid was determined for the 15

TABLE I.
Excretion of Hippuric Acid in Normal Subjects and in Patients with Kidney Dysfunction.

Subject	Weight, lb	First 15-min period		Second 15-min period		Hippuric acid output 30 min., g	Remarks
		Hippuric acid output, g	Urine vol., cc	Hippuric acid output, g	Urine vol., cc		
1	166	.91	49	.48	24	1.39	Normal
		.97	150	.37	18	1.34	
		.93	47	.47	23	1.40	
2	170	.94	134	.33	18	1.27	"
3	175	.87	58	.34	17	1.21	"
4	130	.74	30	.50	16	1.24	"
5	142	.96	146	.47	212	1.43	"
6	135	.25	29	.25	26	0.50	Left hydronephrosis
7	112	.27	31	.26	18	0.53	Hydronephrosis
8	165	.14	18	.40	30	0.54	Pyelonephritis
9	175	.28	86	.53	88	0.81	Chronic prostatitis
10	145	.39	124	.50	120	0.89	Prostatic hypertrophy

The hippuric acid was determined by the senior author's method.³

The urine of subjects 6 to 10 was obtained by catheterization.

¹ Hirsheimer, A., *Am. J. Obs. and Gynec.*, 1939, **37**, 363.

² Smith, F., and Schwei, G. P., *Marquette Med. Rev.*, 1940, **4**, 66.

³ Quick, A. J., *Am. J. Clin. Path.*, 1940, **10**, 222.

and 30 minute periods following the injection of 2.26 g of sodium hippurate.* Typical results are recorded in Table I.

It will be noted that the excretion of hippuric acid in normal men is very rapid. The average output of 15 subjects was 0.88 g in 15 minutes which is 44% of the amount injected. On this basis, an adult can excrete at least 3.5 g of hippuric acid in an hour. Since the average hour output of hippuric acid in a similar group of subjects injected with 1.77 g sodium benzoate was found to be 1.25 g of hippuric acid, one can conclude that the excretory capacity of the kidneys is over 2.5 times as great as the speed of synthesis.

In patients with various types of kidney pathology the excretion of hippuric acid is often definitely diminished. Curiously, the output is frequently considerably higher for the second 15-minute period than for the first, which is difficult to explain by any of the current theories of kidney function. Since the excretion of hippuric acid is diminished in certain renal disorders, it is to be recommended that when liver function is determined by the hippuric acid test in patients with suspected concomitant kidney dysfunction, the excretion rate of hippuric acid should also be estimated.

Summary. The excretion of hippuric acid in normal adults following intravenous injection is exceedingly high: about 44% is eliminated in 15 minutes when 2.26 g of sodium hippurate are administered. In various renal disorders the speed of excretion is diminished.

13785

Restoration of Blood Pressure by Renin Activator After Hemorrhage.†

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That a renin-like substance may be found in the blood stream after hemorrhage was first indicated by Saperstein, Ogden and Southard,¹ who pointed out that a homeostatic mechanism for the regulation of blood pressure might be involved. Independently, similar

* Ampules of sodium hippurate were kindly furnished by George A. Breon, Inc., Kansas City, Mo.

† Aided by a grant from the Board of Research of the University of California.

¹ Saperstein, L. A., Ogden, E., and Southard, F. D., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 505.

conclusions were drawn by Hamilton and Collins,² and more recently by Braun-Menendez,³ in both cases from experiments which clearly demonstrated the renal origin of the substance in question and showed, moreover, that it appeared also in shock and other hypotensive states.

The fact that the renin-like substance is actually renin is indicated first, by its renal origin, and secondly, by the fact that the assay methods of the different groups of workers cited are based on different pharmacological properties characteristic of renin, Braun-Menendez making use of its pressor properties (with added renin activator) and Sapirstein, Ogden and Southard using the effect on the guinea pig ileum with and without added activator.

It appears then that renin is secreted in hemorrhage and perhaps in any acute hypotensive condition.

The fact that the injection of renin into a normal animal will produce tachyphylaxis or exhaustion of activator raised the question whether in prolonged hypotension an animal might, as a result of continued secretion of renin, eventually become tachyphylactic to its own renin; *i. e.*, the substrate (renin activator) on which the enzyme (renin) acted to produce the actual vasoconstrictor (angiotonin) might become exhausted. An experimental basis for this hypothesis is the observation (Ogden, Hildebrand and Page⁴) that in acute renal ischemia the animal becomes progressively less sensitive to renin. This may be explained by the consumption of renin activator by the animal's own renin.

If the organism, by the continued release of renin, exhausts its supply of renin activator in hypotensive states (hemorrhage or shock) then the addition of renin activator to the blood stream of such an animal should exert a pressor effect similar in character to that produced in the normal animal by the injection of renin. The experiments here reported indicate that this is indeed the case.

Preparation of Renin Activator. The substance hereafter referred to in this discussion as renin activator is the serum globulin fraction of ox blood precipitated by bringing to .4 saturation with ammonium sulfate. This is stated to contain renin activator by Page⁵ and also by Braun-Menendez.⁶

² Hamilton, Angie S., and Collins, Dean A., *Am. J. Med. Sci.*, 1941, **202**, 914.

³ Braun-Menendez, E., personal communication to author.

⁴ Ogden, E., Page, E. W., and Hildebrand, G. J., *Proc. Federation Am. Soc. for Exp. Biol.*, 1942, **1**, 63.

⁵ Page, I. H., McSwain, B., Knapp, G. M., and Andrus, W. D., *Am. J. Physiol.*, 1941, **135**, 214.

⁶ Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., and Munoz, J. M., *J. Physiol.*, 1940, **98**, 283.

Fresh beef blood was allowed to clot and the serum removed. The serum was brought to .4 saturation with saturated ammonium sulfate and then supercentrifuged. The precipitate was dissolved in a small amount of distilled water, dialyzed against tap water for 12 hours, made up to 1/10 the original volume of serum used and brought into solution by the addition of 1 g of sodium chloride per 100 cc. The protein concentration of the preparations used ranged from 8-11%. The non-protein nitrogen was negligible.

Experimental. Ten dogs were used in these experiments, 7 under nembutal, 2 under ether and one under chlorotone. A mercury float manometer recorded the carotid blood pressure.

When the normal blood pressure had been recorded, the dog was bled through the femoral artery, 14-21 cc of blood being removed per kilo body weight. The bleeding was stopped and the recording continued until the blood pressure began to show a definite rise. Another 14 cc per kilo was then removed, the process being repeated until the pressure either failed to show recovery or began to fall after a bleeding.

An injection of 10-25 ml of 10% gelatin was then made to control any osmotic or volume effects which might occur incidentally with the subsequent injection of the renin-activator preparation.

The injections of gelatin occasionally produced a slight rise in blood pressure, but this never exceeded 10 mm Hg and was of very brief duration.

After the effect of the gelatin had disappeared, an equal volume of the renin activator preparation described above was injected, a new preparation being used for each dog.

The injection of this preparation, in sharp contrast to the control gelatin solution, produced a very marked rise in blood pressure ranging from 15-80 mm of Hg, which persisted for 20-120 minutes and was succeeded by a gradual fall in pressure (Fig. 1). Further injection of the renin activator preparation during the period of restored blood pressure produced no pressor effect, but after the pressure had fallen to lower levels, whether spontaneously or as the result of further bleeding, renewed injection again restored the blood pressure toward its normal level.

Since renin activator disappears from the blood with time (Page⁷) one renin activator preparation was allowed to stand two weeks before its effect was determined, and another was prepared from serum a week old. Neither of these preparations had any pressor effect; no recovery of blood pressure occurred, and the animals died soon after

⁷ Kohlstaedt, K. G., Page, I. H., and Helmer, O. M., *Am. Heart J.*, 1940, **19**, 42.

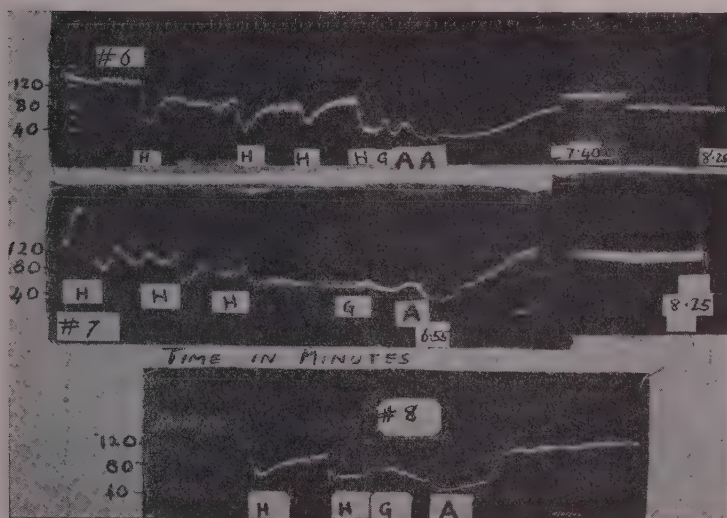


FIG. 1.

The figure shows records of 3 experiments in which successive hemorrhages (H) were followed by injections of gelatin (G), and activator extract (A). For quantities and detailed procedure, see text.

injection. The failure of these two preparations to restore the blood pressure affords another control on the injection of fresh fractions and adds further evidence that our positive findings are, in fact, due to renin activator.

It may be seen in the figure that there was a slight initial drop in blood pressure after the injection of renin activator before the pressor effect appeared. This phenomenon occurred also with the two control preparations just mentioned, and is probably ascribable to foreign-protein effects from ox globulin other than renin activator contained in the preparation.

The pressor effect of the renin activator preparation used in these experiments may be attributed to the fact that the exhausted ingredient of the homeostatic renin-renin activator system had been replenished. In support of this conclusion may be cited the fact that the preparations used had no pressor effect on normal dogs, or dogs whose blood pressure had already been elevated by previous injections of renin activator. In these cases the reno-pressor system was already functioning adequately.

Conclusions. Injection of a renin-activator preparation of ox-plasma restored the blood pressure of dogs after hemorrhage. This restoration was observed with quantities of the preparation containing

only one-tenth or less of the total plasma protein removed.

Corresponding quantities of 10% gelatin or of control plasma concentrations failed to restore the blood pressure.

From these observations it is concluded that the secretion of renin in severe hemorrhage (and perhaps in other forms of shock) is sufficient to produce exhaustion of renin activator. The resulting failure of the reno-pressor system is followed by a fatal collapse of blood pressure. This collapse may be staved off and the blood pressure restored by replacing the exhausted activator by a suitable preparation of ox plasma.

These investigations suggest the possibility of improving the transfusion therapy of hemorrhage and shock by fortifying the plasma with suitable preparations of renin-activator, or possibly by substituting relatively small quantities of activator preparations for plasma in emergency treatment. Work in this direction is in progress.

13786 P

Failure of Heparin to Inhibit Coagulation of Citrated Blood and Plasma in Presence of Staphylococci.*

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Experimental and clinical observations have shown that heparin inhibits the clotting of blood and the formation of thrombi. Recently we have studied the effect of heparin on the coagulation of citrated blood and plasma by staphylococci.¹ Some strains of staphylococci will coagulate human plasma.^{2, 3} One, 0.5 and 0.1 cc of heparin† (Liquamine 1.0 cc = 10 mg) was added to a series of tubes containing 3.0 cc of both rabbit and human plasma and sterile saline (3 parts of plasma and one of saline). One-tenth of a cubic centimeter of a broth culture of staphylococci was added to each of these tubes. The medium in each of the tubes was completely coagu-

* Aided by grants from the John and Mary R. Markle Foundation and the University of Tennessee.

† The heparin was supplied by the Roche-Organon, Inc., of Nutley, New Jersey.

¹ Rigdon, R. H., and Haynes, Anne, to be published.

² Loeb, L., *J. Med. Res.*, 1903, **10**, 407.

³ Chapman, George H., Conrad, Burns, and Merrit, H. Stiles, *J. Bact.*, 1941, **41**, 431.

lated during 24 hours of incubation at 37.5°C. Seven strains of staphylococci (4 aureus, 2 albus and 1 citreus) were then used to study the effect of heparin on the coagulation of plasma. Each of the series of 3 tubes containing the different dilutions of heparin was coagulated by 4 of these 7 strains of staphylococci. Physiological saline in quantities equal to that of heparin was added to a series of tubes of the citrated plasma for the controls. They were inoculated simultaneously with the different strains of staphylococci. Each of the strains of staphylococci that coagulated the heparinized citrated plasma also coagulated the control medium. This citrated plasma medium, containing both heparin and saline, did not coagulate during the time of incubation when staphylococci were not added. When citrated blood was substituted for the plasma, coagulation occurred in a similar manner with the four of the seven strains of staphylococci.

It is evident, therefore, from these observations that heparin, even in a 25.0% concentration, does not inhibit the coagulation of citrated human and rabbit plasma and also blood by the strains of staphylococci containing the so-called coagulase factor.

13787

Bovine Albumin as an Antigen.*

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Minneapolis, Minn.*

The possible use of bovine albumin in man^{1, 2, 3} makes the behavior of bovine albumin as an antigen of some interest. Previous reports^{4, 5, 6} indicate that bovine albumin behaves as a potent antigen

* This work was supported by a grant from Frederick Stearns and Company, Detroit. Valuable assistance was provided by the Work Projects Administration, University of Minnesota Project No. 8760, Sub-project No. 380.

¹ Janeway, C. A., and Beeson, P. B., *J. Clin. Invest.*, 1941, **20**, 435.

² Davis, H. H., Eaton, A. G., and Williamson, J., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 96.

³ Taylor, H. L., Keys, A., and Savage, G., *Proc. Soc. Exp. Biol. and Med.*, 1942, in press.

⁴ Leblanc, A., *La Cellule*, 1901, **18**, 335.

⁵ Hunter, A., *J. Physiol.*, 1905, **32**, 327.

⁶ Hektoen, L., and Welker, W. H., *J. Inf. Dis.*, 1924, **35**, 295.

TABLE I.
Anaphylactic Response of Sensitized Guinea Pigs to Single Intracardial Injections
of Bovine Albumin and Globulin.

	Albumin				Globulin			
Days after inj.	7	14	18	21	7	14	18	21
No. of animals tested	3	3	3	7	3	2	4	2
No. of animals showing symptoms	0	0	3	7	0	2	4	2
No. of animals dying from anaphylaxis	0	0	3	4	0	2	4	1

in rabbits and guinea pigs. This paper is to report some studies on the antigenic behavior of bovine albumin in rabbits, guinea pigs, and in man.

Bovine albumin was prepared as described elsewhere³ by fractionation of plasma with ammonium sulfate. Electrophoretic analysis was carried out by the methods of Tiselius⁷ and Longworth.⁸

Three rabbits were given a course of 6 injections of bovine albumin over a period of 4 weeks. Serum from blood drawn at the end of this period gave a titer as high as 24,000. Sixteen guinea pigs were given a sensitizing dose of 50 mg of albumin per kg and were tested with a shock dose of the same size administered intracardially. Eleven guinea pigs were similarly treated with globulin. All animals which died after the shock dose were posted. The results are given in Table I.

The bovine albumin had a longer sensitization period than globulin. A similar difference has been reported between horse albumin and globulin.⁹

The following case history is definite evidence that bovine albumin may act as an antigen in man. Patient N. R. was given 10 cc of 6% albumin intravenously on November 11, 1939. The skin test to albumin, which had been previously negative, was positive on December 18, 1939, at which time 3 cc of 8.8% albumin solution were administered intravenously over a 5-minute period. At the end of this time the pulse was weak, the respiration was shallow, and cyanosis developed. The injection was stopped. Respiration ceased momentarily but the patient responded to intravenous adrenaline and artificial respiration. After 5 minutes he recovered completely and had no complaints.

A positive skin test does not necessarily indicate that the patient will have anaphylactic symptoms. Four patients with mildly positive skin reactions were given 10 cc of albumin without effect. One

⁷ Tiselius, A., *Faraday Soc.*, 1937, **33**, 524.

⁸ Longworth, L. G., *J. Am. Chem. Soc.*, 1939, **61**, 529.

⁹ Doerr, R., and Berger, W., *Z. Hyg.*, 1922, **96**, 191.

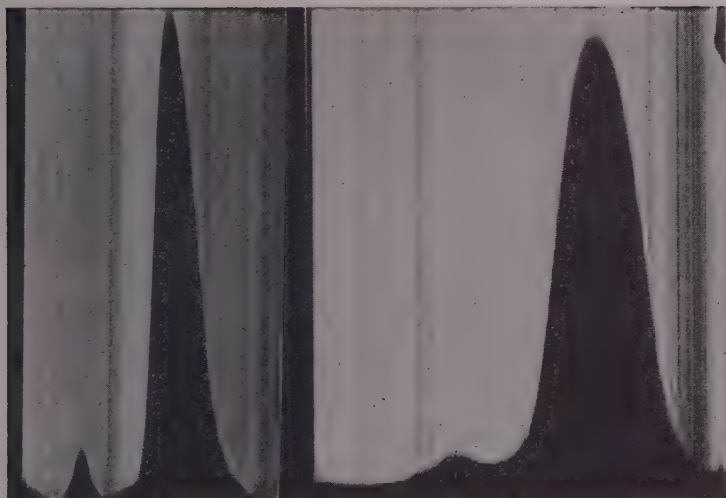


FIG. 1.

Electrophoretic patterns (descending boundary) of bovine albumin in phosphate buffer ionic strength = 0.05 pH = 7.4; left, prepared with ammonium sulfate; right, prepared with methyl alcohol.

patient with a 2+ skin reaction was given 1 cc intravenously followed by the immediate development of urticaria.

Discussion. Representative electrophoretic patterns of the albumin used in this work appear in Fig. 1. A pattern of albumin prepared with methyl alcohol recommended by us⁹ for intravenous use is also included. The antigenic properties of this material have not been studied in detail but it can be expected to give similar results since it has approximately the same electrophoretic pattern and it has been shown¹⁰ that the immunological properties of alcohol precipitated serum protein fractions do not differ from $(\text{NH}_4)_2\text{SO}_4$ treated fractions. Both preparations show 5-7% of a component moving slower than the albumin. Reasons for believing that this slow-moving component does not consist entirely of albumins will be given elsewhere. Some of the anaphylactic properties of these preparations may be due to residual globulin. The work of Svensson,¹¹ Cohn, *et al.*,¹² and our own experience indicates that it is not possible to prepare albumin free of the slower moving component by the simple fractionation procedures which are easily adapted to

¹⁰ Chu, C. T., and Chan, C. G., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 323.

¹¹ Svensson, H., *J. Biol. Chem.*, 1941, **139**, 805.

¹² Cohn, E. J., McMeekin, T. L., Oncley, J. L., Newell, J. M., and Hughes, W. L., *J. Am. Chem. Soc.*, 1940, **62**, 3386.

large-scale production. It is obvious that these procedures are inadequate to prepare non-antigenic albumin. Complete elimination of the slower-moving component may produce a preparation which does not have such striking antigenic properties.

13788

Intravenous Administration of Bovine Plasma Albumin.*

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Minneapolis, Minn.*

Kremen, Taylor, and Hall¹ found that the globulin fraction of bovine plasma produced far more skin reactions than the albumin fraction. Whole bovine plasma produces an alarming number of untoward reactions when administered intravenously to man.² Satisfactory preliminary results with the intravenous administration of the albumin fraction of bovine plasma prepared by ammonium sulphate fractionation have been reported recently.³ The present article is a report on the methods of preparation and results of the intravenous administration of plasma albumin.

Bovine albumin was prepared by fractionation from sterile plasma with (a) ammonium sulfate and (b) methyl alcohol.

Solid ammonium sulfate was dialyzed into sterile plasma through a cellophane membrane at +4°C; 350 g ammonium sulfate were used for each liter of plasma. The albumin was precipitated from the filtrate of the plasma by saturation with ammonium sulfate. The final precipitate was dissolved in distilled water and was dialyzed in collodion bags against a phosphate buffer at pH 7.4, ionic strength = 0.155. The albumin solution was Seitz-filtered, tested for sterility and stored in sealed vials at 4°C until used. Contamination was kept at a minimum by clean technic and main-

* This work was supported by a grant from Frederick Stearns and Company, Detroit. Valuable assistance was provided by the Work Projects Administration, University of Minnesota Project No. 8760, Sub-project No. 380.

¹ Kremen, A. J., Taylor, H. L., and Hall, H., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **42**, 532.

² Wangenstein, O. H., Hall, H., Kremen, A. J., and Stevens, B., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 616.

³ Davis, H. H., Eaton, A. G., and Williamson, J., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **49**, 96.

tenance of temperature at 4°C, but complete sterility during preparation was not achieved except in the final solution.

Pyrogenic response occurred rather frequently when these ammonium sulfate preparations were administered. For comparison, fractionations were made with methyl alcohol and a rigorously controlled aseptic technic. The bacteriological details will be presented elsewhere.⁴ All procedures were carried out in a sterile atmosphere (ultraviolet lamps and air filters). At each stage of preparation samples were taken and cultured at 5°, 25°, and 37°C, both aerobically and anaerobically. If contamination appeared at any stage, the lot was discarded.

Sterile bovine plasma was precipitated in liter centrifuge bottles at -17°C by adjusting the methyl alcohol concentration to 40%. After centrifugation and aseptic transfer of the supernatant liquid, the albumin was precipitated by increasing the methyl alcohol concentration to 70%. This precipitate was washed with 95% and 100% methyl alcohol and 3 times with absolute ethyl ether. It was then dried *in vacuo* and stored as a dry powder until used. For administration the powder was dissolved in sterile pyrogen-free normal saline solution and the solution finally filtered through a Berkefeld N filter.

Large quantities of albumin prepared with ammonium sulfate have been given to dogs, rabbits, and guinea pigs. Two dogs were given one gram of albumin per kilo intravenously without any noticeable effects. Three dogs under pentobarbital sodium anesthesia, with the carotid artery cannulated for blood pressure measurements, were given 0.5 g of albumin per kilo in solutions whose concentrations ranged from 3 to 12% of albumin. No significant change of blood pressure was noted. The blood pressure of one dog was reduced to the shock level of 55 mm Hg by hemorrhage. The blood pressure was restored to its original level by intravenous administration of 100 cc of 10% albumin solution.

The results in man of the intravenous administration of albumin prepared with ammonium sulfate are summarized in Table 1-A. Eight preparations were tested. Four preparations tested with 18 individuals gave no reactions; 4 other preparations tested with 8 individuals gave reactions in 7 cases.

The results of the administration of the methyl alcohol albumin are given in Table 1-B. Five preparations tested in 14 patients gave only 3 reactions; of these, 2 reactions can be attributed to chill-producing substances carried over from bovine plasma.

⁴ Savage, G. M., Taylor, H. L., and Keys, A., in press.

TABLE I.
Summary of Results Obtained with Intravenous Injection of Bovine Albumin.

A Prepared by ammonium sulphate fractionation				B Prepared by methyl alcohol fractionation			
Lot No.	No. patients tested	G albumin adm. per patient	No. of reactions	Lot No.	No. patients tested	G albumin adm. per patient	No. of reactions
12	3	0.7	0	4C ₆	4	3.0	1
15	3	0.5	0	4C ₆	1	3.0	urticaria
17	9	0.7	0	4B ₅	4	3.0	0
22	3	0.5	2	3	3	3.5	1
27	3	9.0	0	4B ₄	2	3.6	0
29	2	0.6	2				
34	2	0.6	2				
41	1	6.0	1				

TABLE II.
Types of Reactions Encountered on Administration of Bovine Albumin.

Patient	Lot No.	Prep. type	G albumin adm. per patient	Immediate reactions 0-30 min	Delayed reactions		
					Max. temp.	Nausea vomiting	Diarrhea
T.C.	22	(NH ₄) ₂ SO ₄	0.5	none	100.8	none	none
C.L.	22	"	0.5	"	100.8	"	"
N.W.	29	"	0.6	"	103	"	"
N.P.	29	"	0.6	"	normal	+	+
E.P.	34	"	0.6	"	102	none	none
W.B.	34	"	0.6	"	104	"	"
N.O.	41	"	6.0	"	—	"	"
E.C.	3	Alcohol	3.5	"	102	"	"
J.Iab.	4C ₆	"	3.0	"	100.8	"	"
A.F.*	4C ₆	"	0.1	urticaria	none	"	"

*Positive skin test 2+.

No significant changes in blood pressure were found during or following the administration of bovine albumin in patients with relatively normal blood pressures at the start. Albumin has been administered in concentrations as high as 15% without influencing blood pressure or pulse rate.

The types of reactions are given in Table II. Severe reactions resulted from the intravenous administration of small amounts of albumin prepared by ammonium sulfate precipitation without proper bacteriological control.

Discussion. The reactions obtained with the intravenous administration of preparations 22, 29, 34, and 41 are attributed in large measure to contamination during preparation and failure to eliminate the resulting pyrogens. The most common contaminants during the winter were found to be cryophilic organisms present on the cattle. These organisms were apparently brought into the laboratory on the bleeding equipment and were found in the ammonium sulfate preparation at a later stage. We have not found it possible consistently to maintain sterile solutions throughout the fractionation of beef plasma with ammonium sulfate.

The advantage of the use of methyl alcohol or other bacteriostatic organic solvents in the preparation of bovine albumin for intravenous use is obvious. Complete control of sterility during the preparation is essential. The ordinary procedures of fractionation do not eliminate pyrogens once they are formed.

The work of Kremen, *et al.*,⁵ suggests that chill-producing substances other than those of bacterial origin may be present in bovine blood. The appearance of two reactions of the chill type in the alcohol preparations which were not contaminated at any time suggests that a single fractionation with methyl alcohol may not completely eliminate the chill-producing substances.

Conclusions. 1. Methyl alcohol is preferable to ammonium sulfate for the preparation of bovine albumin for intravenous use. 2. Bovine albumin can be prepared which gives a low incidence of reactions when administered intravenously to man.

⁵ Kremen, A. J., Hall, H., Koschnitzhe, H. K., Stevens, B., and Wangenstein, O., *Surgery*, 1942, **11**, 333.

Effect of Surface Active Agents on Oxidations of Lactate by Bacteria.

E. J. ORDAL AND A. F. BORG. (Introduced by W. P. Larson.)

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Baker, Harrison and Miller¹ have studied the effect of synthetic surface active agents on the metabolism of bacteria as measured in the Warburg manometric apparatus. Cationic surface active agents were found to inhibit equally the metabolism of both Gram positive and Gram negative bacteria. Anionic agents, in general, inhibit the metabolism of Gram positive bacteria only. In a subsequent paper² studies on bactericidal effects were reported. Dubos³ discussed the differential susceptibility of Gram positive and Gram negative bacteria to different injurious agents, and suggested the use of anionic and cationic surface active agents as tools for the study of bacterial structure.

In this paper we report the effects of an anionic surface active agent, the dioctyl ester of sodium sulfosuccinate, and of a cationic agent, cetyl pyridinium chloride, on a Gram positive organism and a Gram negative organism. As an index of the action of these agents we used the oxidation of lactate by molecular oxygen as compared with anaerobic oxidation by methylene blue.

Methods. Two organisms were used, the Insecticide Board strain of *Staphylococcus aureus*, a Gram positive organism, and *Escherichia coli*, University of Washington No. 406, a Gram negative organism. These two cultures were selected because they possessed relatively active lactic dehydrogenases as determined by the Thunberg technic.

Staphylococcus aureus was grown on the broth prescribed for the Food and Drug Administration procedure for the phenol coefficient.⁴ After 24 hours' incubation, the organisms were collected by means of a continuous flow Sharples centrifuge and washed 3 times with M/100 phosphate buffer at pH 7.0. *Escherichia coli* was grown on Blake bottles containing F. D. A. broth to which 2% agar had been added. The organisms were collected and then washed as described above for *Staphylococcus aureus*.

¹ Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **73**, 249.

² Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **74**, 611.

³ Marshall, E. K., Jr., Loekwood, J. S., and Dubos, R. J., *Chemotherapy*, Philadelphia, University of Pennsylvania Press, 1941.

⁴ Circular No. 198, 1931, U. S. D. A.

TABLE I.
Effect of Surface Active Agents on Lactic Dehydrogenases in *Staphylococcus aureus* and *Escherichia coli*.

	<i>Staphylococcus aureus</i>				<i>Escherichia coli</i>				
Conc. of OT, moles per l	0	1.6x 10 ⁻⁴	3.2x 10 ⁻⁴	6.4x 10 ⁻⁴	0	5.3x 10 ⁻⁴	2.1x 10 ⁻³	2.5x 10 ⁻²	5.1x 10 ⁻²
Reduction time of methylene blue in min.	3	6	33	60	4	5	11	33	60
Conc. of CPR in moles per l	0	4.3x 10 ⁻⁶	8.6x 10 ⁻⁶	1.7x 10 ⁻⁵	0	3.4x 10 ⁻⁵	1.4x 10 ⁻⁴	5.5x 10 ⁻⁴	11.1x 10 ⁻⁴
Reduction time of methylene blue in min.	3	3	11	60	4	5	13	38	60

OT—dioctyl ester of sodium sulfosuccinate.

CPR—cetyl pyridinium chloride.

The activity of lactic dehydrogenases in the cell suspensions was determined by the modified Thunberg method of Ordal and Halvorson⁵ with methylene blue as indicator. Each Thunberg tube contained 5 ml M/15 phosphate buffer at pH 7.56, 0.25 ml 0.2 M sodium lactate, 1 ml 1-5000 methylene blue, 0.25 ml cell suspension and 2 ml H₂O or surface active agent. Tests for dehydrogenase activity were terminated at 60 minutes, and in general, controls without substrate showed no signs of decolorization at this time. All experiments were performed at 37°C.

The effect of the presence of varying concentrations of the dioctyl ester of sodium sulfosuccinate and of cetyl pyridinium chloride on the activity of the lactic dehydrogenases of *Staphylococcus aureus* and of *Escherichia coli* is shown in Table I.

The oxidation of lactate by oxygen was determined in conventional Warburg manometers. The final concentrations of phosphate, lactate and cell suspensions were identical with those used in the

TABLE II.
Oxygen Uptake from Lactate Solutions by Suspensions of *Staphylococcus aureus* in the Presence of Surface Active Agents.

Surface active agent	Conc. in moles/l	Oxygen uptake μ l at			
		15 min	30 min	60 min	120 min
None		8	17	34	60
OT	8.0 x 10 ⁻⁵	7	17	33	57
"	1.6 x 10 ⁻⁴	6	11	24	43
"	3.2 x 10 ⁻⁴	2	5	10	22
"	6.4 x 10 ⁻⁴	—1	3	6	10
CPR	2.2 x 10 ⁻⁶	7	14	27	42
"	4.3 x 10 ⁻⁶	6	12	17	21
"	8.6 x 10 ⁻⁶	1	3	6	7

OT—dioctyl ester of sodium sulfosuccinate.

CPR—cetyl pyridinium chloride.

⁵ Ordal, E. J., and Halvorson, H. O., *J. Bact.*, 1939, **38**, 199.

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TABLE III.
Oxygen Uptake from Lactate Solutions by Suspensions of *Escherichia coli* in the Presence of Surface Active Agents.

Surface active agent	Conc. in moles/l	Oxygen uptake in μ l at				
		15 min	30 min	45 min	60 min	75 min
None		35	71	103	132	160
OT	2.5×10^{-2}	33	67	94	118	142
"	5.1×10^{-2}	31	65	90	111	130
CPR	1.1×10^{-3}	28	56	84	103	122
"	2.2×10^{-3}	12	26	36	40	42
"	4.5×10^{-3}	2	7	9	11	11

OT—dioctyl ester of sodium sulfosuccinate.

CPR—cetyl pyridinium chloride.

Thunberg tubes. The results of tests with varying concentrations of the surface active agents are shown in Tables II and III.

Results. It is apparent from the data shown in Table I that the lactic dehydrogenase of *Staphylococcus aureus* is far more susceptible to the action of both surface active agents than that of *Escherichia coli*. However, the inhibitory effect of the surface active agents disappears quickly with dilution with *Staphylococcus aureus* while with *Escherichia coli* there is a considerable range of concentrations in which there is a relatively slow change in inhibition. In particular, the lactic dehydrogenase of *S. aureus* is inhibited by 1.7×10^{-5} M cetyl pyridinium chloride. On dilution to one-fourth this concentration, the inhibitory effect disappears. On the other hand, a concentration of 1.1×10^{-3} M cetyl pyridinium chloride is required to inhibit the lactic dehydrogenase of *E. coli*, and a dilution in excess of 33 times is necessary before all traces of inhibition disappear.

Examination of the data in Tables II and III, shows that both surface active agents inhibit the oxidation of lactate by *S. aureus*, but that only the cationic agent inhibits the oxidation of lactate by *E. coli*.

For convenience in comparing the susceptibility of the two organisms, we have designated as "critical" concentrations those concentrations of surface active agents inhibiting the reduction by methylene blue for 60 minutes in the tests for dehydrogenases, and the concentrations giving nearly complete inhibition after 60 minutes in the tests for oxygen uptake. These data are collected in Table IV.

In the case of *S. aureus*, there is little difference between the concentrations of surface active agents required to inhibit oxidation of lactate by oxygen, as compared to oxidation by methylene blue. Presumably, therefore, the cytochrome systems normally concerned in the oxidation of lactate by oxygen in *S. aureus* possess no special

TABLE IV.
 "Critical" Concentrations of Surface Active Agents Inhibiting Oxidation of
 Lactate by Oxygen and by Methylene Blue.

Surface active agent	Oxidation by	Organism	
		<i>Staph. aureus</i>	<i>E. coli</i>
Dioctyl ester of sodium sulfosuccinate	methylene blue oxygen	$6.4 \times 10^{-4}M$	$5.1 \times 10^{-2}M$
		$6.4 \times 10^{-4}M$	—*
Cetyl pyridinium chloride	methylene blue oxygen	$1.7 \times 10^{-5}M$	$1.1 \times 10^{-3}M$
		$8.6 \times 10^{-6}M$	$4.5 \times 10^{-5}M$

*A concentration of $5.1 \times 10^{-2}M$ only slightly slowed down the oxidation of lactate by oxygen (See Table III).

sensitivity to these surface active agents. Anson⁶ has shown that synthetic surface active agents are extremely active in the denaturation of proteins. It seems likely that the proteins of *S. aureus* concerned in the oxidation of lactate by methylene blue are relatively accessible to the surface active agents.

There are striking differences in the effects of the surface active agents on the oxidations of lactate by *E. coli*. Since the oxidation of lactate by molecular oxygen is inhibited by a lower concentration of cetyl pyridinium chloride than oxidation by methylene blue, it would appear either that the cytochrome systems peculiar to *E. coli* are more sensitive to the surface active agent than the other proteins concerned in the oxidation of lactate, or are more available to the action of the agent.

The anomalous result with the dioctyl ester of sodium sulfosuccinate, inhibition of methylene blue reduction by a $5.1 \times 10^{-2}M$ concentration, a concentration which affects only slightly the oxidation of lactate by oxygen, is most logically due to the reduced availability to the cells of the methylene blue due to the high concentration of the surface active agent which is present in this concentration largely in the form of micelles.

The enzymes of the two organisms which are concerned in the oxidation of lactate, differ profoundly in sensitivity to the anionic surface active agent. This is in accord with the results of Baker, Harrison and Miller.^{1, 2}

Dubos³ has pointed out that Gram negative bacteria contain so-called lipo-polysaccharide antigens which appear to form an outer coating on the cells, and Baker, Harrison and Miller⁷ have shown that certain phospholipids protect bacterial cells against the action of both cationic and anionic surface active agents. It is possible,

⁶ Anson, M. L., *J. Gen. Physiol.*, 1939, **23**, 239.

⁷ Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **74**, 621.

therefore, that a cell membrane containing substances of this character in the Gram negative bacteria may account for the lack of sensitivity to the anionic agent. We are, therefore, attempting to prepare cell-free enzymes from *S. aureus* and *E. coli* in order to determine the rôle played by the cell membranes in the results shown above.

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Sulfonamide-Resistant Strains of Staphylococci: Clinical Significance.*

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Strains of pneumococci, gonococci, *E. coli* and *S. aureus* have been rendered resistant *in vitro* to the bacteriostatic action of sulfonamide drugs.¹⁻⁶ Rammelkamp⁷ has also observed that *S. aureus* may become "fast" to tyrothricin, both *in vitro* and *in vivo*. The present report is concerned with the *in vitro* and *in vivo* production of sulfonamide resistant strains of staphylococci.

The *in vitro* observations were made with 2 strains of *S. aureus* isolated from patients with severe staphylococcal infections. A chemically-defined medium as described by Gladstone was used throughout.⁸ These strains were made resistant by exposure to increasing concentrations of sodium sulfathiazole in this medium. Initial growth was established with a minute inoculum from an agar slant. Subsequent transfers of the cultures in the synthetic medium were made with a 5 mm wire loop. The two strains showed an initial difference in susceptibility to the action of sulfathiazole. Strain 14

* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

¹ MacLean, I. H., Rogers, K. B., and Fleming, A., *Lancet*, 1939, **1**, 562.

² MacLeod, C. M., and Doddi, G., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 69.

³ Lowell, F. C., Strauss, E., and Finland, M., *Ann. Int. Med.*, 1940, **14**, 1001.

⁴ Westphal, L., Charles, R. L., and Carpenter, C. M., *Ven. Dis. Inform.*, 1940, **21**, 183.

⁵ Strauss, E., Dingle, J. H., and Finland, M., *J. Immunol.*, 1941, **42**, 313.

⁶ *Ibid.*, 1941, **42**, 331.

⁷ Rammelkamp, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 346.

⁸ Gladstone, G. P., *Brit. J. Exp. Path.*, 1939, **20**, 189.

grew readily for several generations in the presence of 0.25 mg per 100 ml of sulfathiazole, but strain 7 was unable to institute growth in this concentration after the second transfer. It was necessary to start strain 7 in 0.1 mg per 100 ml. Control observations were carried out with the parent strains transferred an equal number of times in a drug-free medium.

The degree of growth was estimated regularly by measuring the density of the culture in the Evelyn photoelectric colorimeter, as previously described.⁹ The development of drug resistance was determined by seeding approximately 1,000 organisms per ml to tubes of medium containing varying concentrations of sulfanilamide, sulfapyridine, sulfadiazine, and sulfathiazole. Drug resistance was determined by a comparison of growth at the end of 24 and 70 hours of the parent strain and the "fast" strain in the presence of the foregoing drugs.

After 130 transfers in increasing concentrations of sodium sulfathiazole, that is, up to 10 mg per 100 ml, strain 14 grew readily in the presence of 200 mg per 100 ml of sodium sulfapyridine, 100 mg per ml of sodium sulfadiazine, and 50 mg per 100 ml of sodium sulfathiazole. Strain 7 was transferred an equal number of times in increasing concentrations of sodium sulfathiazole up to 5 mg per 100 ml. After this, maximum growth took place in the presence of 200 mg per 100 ml of sodium sulfapyridine, 75 mg per 100 ml of sodium sulfadiazine, and 40 mg per 100 ml of sodium sulfathiazole. Studies are now in progress to determine whether the biologic activity of the "fast" strains differs from the parent strains.

To investigate the *in vivo* development of resistance to sulfonamide action, cultures were made from two patients with staphylococcal bacteremia before and after they had received sulfathiazole. Drug resistance was determined as described for the *in vitro* "fast" strains.

Two strains were secured from a patient having a maximum of 260 colonies per ml of blood, and complicated by the appearance of a retrobulbar abscess.[†] The patient recovered completely. Strain 116a was isolated from the blood stream at the onset of the illness. The patient was then treated with sulfathiazole, parenterally and orally. Subsequent blood cultures remained sterile, but the localized lesion was treated by daily irrigation with sulfathiazole solution. Strain 116b was cultured from this lesion 11 weeks after isolation of 116a. Strains 117a and 117b were obtained from the blood of a

⁹ Spink, W. W., and Jermsta, J., *Proc. Soc. Exp. Biol. and Med.* 1941, **47**, 395.

[†] This patient was observed through the courtesy of Dr. Jay C. Davis of Minneapolis.

TABLE I.
In Vivo Development of Sulfonamide-Resistant *S. aureus*.

Strain	Incubation hr	Concentration of drug in mg per 100 ml											
		Sulfathiazole				Sulfadiazine				Sulfapyridine			
		10	5	2	1	10	5	2	1	10	5	2	1
116a	24	0	0	0	0	0	0	0	0	0	0	0	0
	70	0	0	0	0	0	0	0	++	0	0	+	++
116b	24	0	0	+	++	+	++	++	++	+	+	++	++
	70	0	+	++	++	++	++	++	++	++	++	++	++
117a	24	0	0	0	0	0	0	0	0	0	0	0	+
	70	0	0	0	0	0	0	++	++	0	0	++	++
117b	24	0	0	+	++	+	++	++	++	+	++	++	++
	70	++	++	++	++	++	++	++	++	++	++	++	++

a = Isolated before sulfathiazole therapy.

b = Isolated after sulfathiazole therapy.

+

++ = Maximum growth.

second patient having a maximum of 500 colonies per ml of blood. This patient expired. Strain 117b was isolated after 8 days of sulfathiazole therapy, and 3 days prior to death. While under treatment, the sulfathiazole concentration in the blood was 15 mg per 100 ml.

The results of the tests for drug resistance are recorded in Table I. Both strains became markedly resistant to the action of the sulfonamide compounds.

To our knowledge, these observations constitute the first recorded instance of staphylococci becoming resistant to the bacteriostatic action of the sulfonamides in the human body. It is not unlikely that further studies under way may reveal similar cases, and this phenomenon may explain, in part, the failure of sulfathiazole to clear the blood stream of organisms. It is significant that in the case of the patient who expired, coincident with the development of a "fast" strain of staphylococcus, there was a marked increase in the number of colonies in the blood. This "fastness" was detected after only 8 days of therapy.

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Evaluation of Acetylation of Sulfanilamide as a Test of Liver Function.*

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From the Mayo Foundation, Rochester, Minn.

In a study of the excretion of sulfanilamide by the digestive glands the possibility was considered that a test of liver function might be evolved.¹

It is a known fact that in certain animals, including man, the body detoxicates aromatic amines by means of acetylation. This is the mechanism employed in the case of sulfanilamide.^{2,7} This process has been studied extensively by investigators using *p*-amino-benzoic acid and sulfanilamide, and evidence presented would indicate that the liver is the sole organ in which this chemical change takes place.^{2,8}

As sulfanilamide in the urine is excreted in a state of only partial acetylation in man,³ it was thought that varying percentages in the amount of the acetylated drug might reflect the state of liver function.

In accordance with this idea, small amounts of sulfanilamide were given to normal subjects and to subjects known by accepted tests of liver function and by other means such as peritoneoscopy and surgical exploration to have hepatic disease. In some cases the diagnosis of hepatic disease was confirmed later at necropsy. The amounts of sulfanilamide were considered to be sufficiently small not to cause any injurious results in the subjects tested. Urine was collected for 24 hours following the administration of the drug. Samples from this urine

* This work was started in the Northwestern University Medical School laboratories of Dr. A. C. Ivy. It was completed in the clinical pathology laboratories of the Mayo Clinic.

1 Carryer, H. M., and Ivy, A. C., *J. Pharm. and Exp. Therap.*, 1939, **66**, 302.

2 Ellinger, Alexander, and Hensch, Marie, *Z. f. physiol. Chem.*, 1914, **91**, 21.

3 Ambrose, A. M., and Sherwin, C. P., *Ann. Rev. Biochem.*, 1933, **2**, 377.

4 Sherwin, C. P., *Physiol. Rev.*, 1922, **2**, 238.

5 Muenzen, J. B., Cerecedo, L. R., and Sherwin, C. P., *J. Biol. Chem.*, 1926, **67**, 469.

6 Harrow, Benjamin, and Sherwin, C. P., *Ann. Rev. Chem.*, 1935, **4**, 263.

7 Harrow, Benjamin, Power, F. W., and Sherwin, C. P., *Proc. Soc. Exp. Biol. AND MED.*, 1927, **24**, 422.

8 Harris, J. S., and Klein, J. R., *Proc. Soc. Exp. Biol. AND MED.*, 1938, **38**, 78.

9 Marshall, E. K., Jr., Cuning, W. C., and Emerson, Kenneth, Jr., *Science*, 1937, **85**, 202.

were analyzed for their percentage content of acetylated and non-acetylated sulfanilamide by the method of Marshall and his associates.^{10, 11} Portions of urine were hydrolyzed and analyzed in duplicate.

Nineteen healthy subjects were given from 5 to 30 grains (0.3 to 2 g) of sulfanilamide. The drug was given from one to 6 times to the various subjects. * Analysis of the following 24-hour specimen of urine revealed that from 40 to 59% of the drug was acetylated, the average being 48%. Sixteen patients, 15 of whom had an elevated concentration of serum bilirubin with a direct van den Bergh reaction resulting from portal cirrhosis, disease of the biliary tract or carcinoma of the pancreas, were given 15 grains (1 g) of the drug. In these patients from 21 to 61% of the drug was acetylated. The data obtained did not correlate with the severity of the disease as indicated by the serum bilirubin, bromsulfalein elimination, prothrombin time and hippuric acid elimination tests. Five other patients, who did not have jaundice but did have cardiac cirrhosis of the liver (one patient), carcinomatosis (2 patients) or severe thyrotoxicosis (2 patients), were given 15 grains (1 g) of the drug. These patients acetylated normal amounts of the drug.

It was evident that the proposed test did not have any definitely apparent value in the determination of liver function or in the detection of hepatic disease. A possible explanation for the failure of this test may be found in the fact that ingestion of relatively small amounts of several substances will increase acetylation in the liver greatly. These substances include many of the amino acids, ethyl ester of diacetic acid, sodium acetate, beta-oxybutyric acid, pyruvic acid, glyceric aldehyde, glycerol, acetic aldehyde, and other compounds. It is probable that the metabolism of many of these is changed greatly with advanced liver disease.

Also, it has been shown recently that the body can deacetylate certain compounds.¹²

Summary. The extent to which sulfanilamide is acetylated by the liver was found not to be of any definite value as a test of liver function.

¹⁰ Marshall, E. K., Jr., Cutting, W. C., and Emerson, Kendall, Jr., *J. A. M. A.*, 1938, **110**, 252.

¹¹ Marshall, E. K., Jr., and Litchfield, J. T., Jr., *Science*, 1938, **88**, 85.

¹² Henderson, E., (Schering Corporation), personal communication to Northey, E. H.; *Chem. Rev.*, 1940, **27**, 85.

13792 P

Reactions of Monkeys to Experimental Respiratory Infections.
V. Hematologic Observations in Nutritional Deficiency States.*

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Earlier workers have reported studies on the hematology of vitamin B deficient monkeys.^{1, 2} The current observations were undertaken in an attempt to determine which, if any, of the presently recognized components of the vitamin B complex will maintain or restore specific blood cell and nutritional equilibria.

Methods. Healthy *Macaca mulatta*, receiving a normal diet, were isolated in individual cages and observed clinically and hematologically for a control period of 2 to 3 weeks. The animals showing satisfactory equilibria were then divided into 3 groups and placed on experimental diets: (1) No. 600¹ (modified Goldberger), (2) No. 1 and (3) No. 2 (Table I). The controls have included observations on each experimental animal before it was placed on diet and on a number of monkeys of the same species and approximate age maintained on stock diet. These animals showed no hematologic changes comparable to those to be described.

Results. All of the monkeys on the experimental diets developed leucopenia. In the 4 animals on diet 600, this appeared between the 42nd and 77th days. In the 6 animals receiving diet 1 and in the 5 receiving diet 2, the time of appearance of the initial leucopenia varied between the 30th and 103rd days.

One interesting feature of the response to these diets was a "plateau" period of remarkably stabilized total white cell counts preceding the first appearance of leucopenia. The relative constancy of the total white cell counts between the 14th and 45th diet days seen in monkey No. 4 (Fig. 1) is representative. Similar well-defined periods of stability varying in duration from 24 to 100 days were seen in 3 of the 4 monkeys on diet 600, 4 of the 6 on diet 1, and 2 of the 5 on diet 2.

* This work has been aided by a grant from the International Health Division of the Rockefeller Foundation. Constituents of the special diets were generously furnished by the S.M.A. Corporation.

¹ Langston, W. C., Darby, W. J., Shukers, C. F., and Day, P. L., *J. Exp. Med.*, 1938, **68**, 923.

² Day, P. L., Langston, W. C., Darby, W. J., Wohlin, J. G., and Nims, V., *J. Exp. Med.*, 1940, **72**, 463.

TABLE I
Diet No. 1

Basic Diet*	%	Vitamin Supplements†	
		Daily dosage,	mg
Sucrose	68	Thiamine Hydrochloride	1
Vitamin-Free Casein	18	Riboflavin	1
Vegetable Oil	8	Peridexin Hydrochloride	1
Salt Mixture U.S.P. No. 2	4	Nicotinic Acid Amide	25
Cod Liver Oil U.S.P.	2	Calcium Pantothenate	3
	—	Ascorbic Acid	25
	100		

Diet No. 2

Diet No. 1 with addition of:†

	Daily dosage, mg
Choline Chloride	50
Fumaric Acid	1
Glutamine	1
Inositol	1
Sodium pyraminidopantoate	50

* Mineral mixtures in the basic diet and water were available to the animals *ad libitum*.

† Vitamins B₁₂ and B₆ in suspensions suspended in water and fed by stomach tube once a week.

‡ Erythrocyte counts were done on feces of some of these animals with and without addition of B₁₂ to the diet. Since these assays indicated synthesis of large amounts in the intestine of the monkey B₁₂ supplements were discontinued.

Characteristic of the development of the leucopenia was an initial transitory decrease in circulating white cells, followed by one or more periods of temporary recovery, alternating with progressively more severe leucopenic depressions. The profound terminal cytopenia involved all of the white cell elements. Thus, on the 48th diet day, monkey No. 4 showed a fall to 7000 white cells from the lowest normal level of 13,500, while the lymphocytes were reduced from 5300 to 3700 and the neutrophils from 4500 to 1800. Following this initial leucopenia there was a period of recovery to normal levels until the 70th day of diet, when the lymphocytes were reduced to the previous leucopenic level and the neutrophils and monocytes fell to still lower levels. Another brief period of partial recovery preceded the final precipitous fall of all the white cell elements (2000 W.B.C.) with death on the 91st diet day (Fig. 1).

The leucopenia was more consistent than, and independent of, the anemia. Three of the 4 monkeys receiving diet 600 exhibited moderate degrees of anemia. There was no significant anemia prior to the terminal state in 3 of the 6 monkeys receiving diet 1 (Nos. 22, 26, 27), nor in 3 of the 5 receiving diet 2 (Nos. 16, 40, 42). Monkey No. 23 (diet 1) and monkey No. 136 (diet 2) both showed moderate degrees of normocytic anemia appearing between the 70th

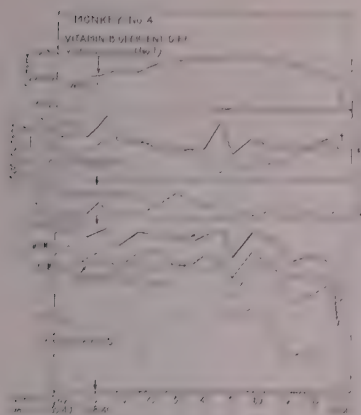


Fig 1

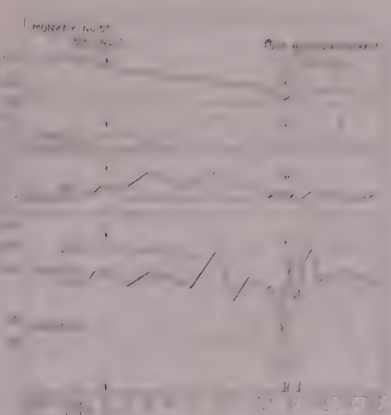


Fig 2

and 82nd diet days. Two of the monkeys on diet 600 (Nos. 2, 14) and 2 of those on diet 1 (Nos. 4, 11) exhibited slight microcytic anemia, appearing in each case on the 33rd or 34th diet day and lasting for 38 to 40 days.

Two monkeys, exhibiting leucopenia while on diet² with *perone* vitamin supplements as indicated were given intramuscular injections of folic acid concentrate (S.M.A.) prepared from yeast autolysate.³ Preliminary observations (*e. g.*, monkey No. 53, Fig. 2) suggest that some factor in this preparation is effective in reestablishing a normal white cell equilibrium. In striking contrast (in the following paper,⁴ Fig. 1) there was prompt exhaustion of a transitory abortive leucocytic response to infection during a similar period of nutritional leucopenia.

³ Hutchings, B. L., Bohonos, N., Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 561.

⁴ Saslaw, S., Schwab, J. L., Woolpert, O. C., Wilson, H. E., *in press*.

Nature of Immunity in Poliomyelitis.

A. F. RASMUSSEN, JR., AND PAUL F. CLARK.

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The evidence increases that circulating antibodies play a minor part in the resistance to poliomyelitis both in man and in monkeys. Antisera have commonly failed to prevent the disease¹ even when used in large quantities or to modify its course when used therapeutically;² the disease has developed both in monkeys and in man even though they possessed neutralizing blood of high titer;³ antibodies have been found in 75 to 85% of normal adults regardless of previous poliomyelitis;⁴ neither resistance nor susceptibility to the disease in man or monkey presents any close correlation to the presence or absence of demonstrable antibodies.⁵ In consequence of these findings, some authors have attempted to seek an answer to the problem of immunity in this disease on an hormonal basis,⁶ with conflicting results, others have raised the query of nutritional deficiencies,⁷ while still others have employed the term "tissue immunity"⁸ to indicate the resistance, due to obscure factors, apparently independent of humoral antibodies. In attempting to unravel the varying reports on this type of resistance, one must bear in mind the primary cellular basis of immunity and anaphylaxis, the many instances of interference phenomena⁹ sometimes entirely non-specific, as well as the possibility of closely bound antibodies such as those demonstrated with the Shope rabbit papilloma virus.¹⁰

* Aided by grants from The National Foundation for Infantile Paralysis, Inc., and from Jeremiah Milbank.

¹ Schultz, E. W., and Gebhardt, L. P., *J. Pediat.*, 1935, **6**, 615; 1935, **7**, 332.

² Kramer, S. D., Aycock, W. L., Solomon, C. I., and Thenebe, C. L., *New England J. Med.*, 1932, **206**, 432; Park, W. H., *J. A. M. A.*, 1932, **99**, 1050.

³ Harmon, P. H., and Harkins, H. N., *J. A. M. A.*, 1936, **107**, 552; Howitt, B. F., *J. Infect. Dis.*, 1937, **60**, 113; Kessel, J. F., and Stimpert, F. D., *J. Immunol.*, 1941, **40**, 61; Harmon, P. H., Harkins, H. N., Fahey, J. J., and Wasbotten, P. M., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 585; Brodie, M., Fiseher, A. E., and Stillerman, M., *J. Clin. Invest.*, 1937, **16**, 447.

⁴ Schaeffer, M., and Muckenfuss, R. S., Lancaster, Science Press, 1940; Shaughnessy, H. J., Harmon, P. H., and Gordon, F. B., *J. Prev. Med.*, 1930, **4**, 463; Howitt, B. F., *J. Infect. Dis.*, 1932, **51**, 565.

⁵ Harmon, P., and Harkins, H. N., *loc. cit.*; Harmon, P. H., Harkins, H. N., Fahey, J. J., and Wasbotten, P. M., *loc. cit.*; Brodie, M., Fiseher, A. E., and Stillerman, M., *loc. cit.*; Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, 1936, **64**,

Recently Howe and Bodian¹¹ have summarized their extensive investigations on neural mechanisms in poliomyelitis in a telling monograph. Closely related to the problem of our paper is their demonstration that a second attack of poliomyelitis can be induced in rhesus monkeys either with homologous or heterologous strains of virus provided a different portal of entry is employed, thus bringing the virus in contact with nervous tissue not previously involved with the virus. They suggest that their experimental data support the conception "that immunity to poliomyelitis in man is not the result of immunization of the nervous system but rather some process which prevents infective quantities of active virus from reaching nervous tissue."

We have been interested in determining what difference, if any, could be shown in the persistence and propagation of poliomyelitis virus along peripheral nerves of strongly immune as compared with normal monkeys. A preliminary paper on this point was presented at a meeting of the Society of American Bacteriologists in 1941.¹²

Four rhesus monkeys, which had recovered from a paralytic attack of poliomyelitis with MV virus and had resisted a second intracere-

739; Shaughnessey, H. J., Harmon, P. H., and Gordon, F. G., *J. Prev. Med.*, 1930, **4**, 463; Harmon, P. H., *Am. J. Dis. Child.*, 1934, **47**, 1179; Stewart, F. W., and Rhoads, C. P., *J. Exp. Med.*, 1929, **49**, 959.

⁶ Draper, G., New York, Appleton-Century, 1935; Levine, M. I., Neal, J. B., and Park, W. H., *J. A. M. A.*, 1933, **100**, 160; Thelander, H. E., and Pryor, H. B., *Arch. Pediat.*, 1933, **50**, 749; Jungeblut, C. W., and Engle, E. T., *J. Exp. Med.*, 1934, **59**, 43; Hudson, N. P., Lennette, E. H., and King, E. Q., *J. Exp. Med.*, 1934, **59**, 543; Aycock, W. L., *Am. J. Pub. Health*, 1937, **27**, 575; Aycock, W. L., Harvard Univ. Pr., 1940, p. 555; Wiraboff, A., *Rev. de laryng.*, 1934, **55**, 896; Reynolds, S. R. M., and Foster, F. I., *Am. J. Physiol.*, 1940, **131**, 422.

⁷ Jungeblut, C. W., *J. Exp. Med.*, 1937, **65**, 127; Jungeblut, C. W., *J. Exp. Med.*, 1939, **70**, 315; Sabin, A. B., *J. Exp. Med.*, 1939, **69**, 507; Heaslip, W. G., *Australian J. Exp. Biol. and M. Sc.*, 1938, **16**, 287; McCormick, W. J., *Canad. M. A. J.*, 1938, n.s. **38**, 260; Toomey, J. A., *Am. J. Dis. Child.*, 1937, **53**, 1202.

⁸ Hudson, N. P., Lennette, E. H., and Gordon, F. B., *J. A. M. A.*, 1936, **106**, 2037; Lennette, E. H., and Hudson, N. P., *J. Infect. Dis.*, 1939, **65**, 78; Goodpasture, E. W., Douglas, B., and Anderson, K., *J. Exp. Med.*, 1938, **68**, 891; Parker, R. F., *J. Exp. Med.*, 1938, **67**, 361; Hodes, H. L., and Webster, L. T., *J. Exp. Med.*, 1938, **68**, 263; Burnet, F. M., and Lush, D., *Lancet*, 1939, **1**, 629; Anderson, K., *Science*, 1939, **90**, 497; Lurie, M. B., *J. Exp. Med.*, 1942, **75**, 247.

⁹ Findlay, G. M., and MacCallum, F. O., *J. Exp. Med.*, 1937, **44**, 405; Theiler, M., *J. Exp. Med.*, 1937, **65**, 705; Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 79; Dalldorf, G., Douglass, M., and Robinson, H. E., *J. Exp. Med.*, 1937, **67**, 333.

¹⁰ Kidd, J. G., *J. Exp. Med.*, 1939, **70**, 583.

¹¹ Howe, H. A., and Bodian, D., *Neural Mechanisms in Poliomyelitis*, The Commonwealth Fund, New York, 1942.

¹² Rasmussen, A. F., Jr., and Clark, Paul F., *J. Bact.*, 1941, **42**, 143.

bral inoculation, were available. The other immune animals were obtained by inoculating a mild "Kessel strain" (McK) intracerebrally into rhesus monkeys which had been used previously, but which had not shown signs of disease. The animals recovering from infection with this strain were then reinoculated with MV virus, the result in most instances being a second attack with more complete paralysis or paralysis of limbs not previously involved. The survivors were used in these experiments.

Inoculations were made into the sciatic nerve just above the knee, excepting in monkey 419, series IV, in which the muscles of the leg were so atrophied that the ulnar nerve at the elbow was used. In each instance, the sciatic nerve employed was supplying functional muscle groups. In series I, 0.5 ml of 5% virus suspension was injected at several points so that the nerve sheath ballooned markedly. After it became evident that this method did not insure consistent infection, satisfactory results were obtained by the method of Bodian and Howe;¹³ the nerve was sectioned with sharp scissors, and the proximal end soaked in 5% virus suspension for 4-10 minutes. Two to 5 days after inoculation, the immune animals and the similarly inoculated controls were sacrificed, and 0.5 to 2 g portions of cervical cord, lumbar cord, sciatic nerve proximal to the sciatic notch, and sciatic nerve at the site of inoculation were removed aseptically with separate sets of sterile instruments. Each tissue was then tested for virus by injecting 1.0 ml of a 10% suspension into the brains of normal monkeys. The effectiveness of the intrasciatic inoculation and the resistance of the immune monkeys was determined by using normal and immune animals in each series as controls. (For details, see Table I.)

In series I, in which the method of inoculation was ineffective, only one of the tissues reinjected, the sciatic nerve at the site of the inoculation of the normal control, Monkey 412, contained demonstrable virus. In the remaining experiments, Series II, III, and IV, in which the "nerve soak" method was used, the normal controls were paralyzed in from 4 to 5 days, while the immune controls were uniformly resistant to infection. In the normal animals which were inoculated and later sacrificed, the virus was demonstrated at progressively higher levels as time advanced and the lesions developed; the virus was never found in the immune animals.

Evidence of virus invasion as seen by microscopic examination of sections of the cords of the animals sacrificed in Series IV agreed essentially with the animal titrations, no sign of recent virus activity

¹³ Bodian, D., and Howe, H. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **44**, 170.

TABLE I.

Tissues of sacrificed animals reinjected (1 ml of 10% suspension intracerebrally)							
Series	Monkey No.	Method of inoculation (M.V. virus)	Subsequent course	Cervical cord	Lumbar cord	Sciatic nerve	Sciatic nerve
						prox. to sciatic notch	at site of inoculation
I	167 Immune	0.5 ml 5% susp. inj. into sciatic nerve	Sacrificed 2 days		417 N P*	415 N P	410 N P
	412 Normal		" "		418 N P	416 N P	411 P+ 11 days
	223 Immune		Control N P				
	413 Normal		" N P				
II	421 Immune	Prox. end of sectioned sciatic nerve soaked in 5% susp. 4 min.	Sacrificed 5 days	470 N P	469 N P	468 N P	450 N P
	464 Normal		" "	467 N P	466 P 9 days	465 N P	449 N P
	419 Immune		Control N P				
	459 Normal		" P 5 days				
III	431 Immune	Prox. end of sectioned sciatic nerve soaked in 5% susp. 10 min.	Sacrificed 5 days	439 N P	438 N P	437 N P	434 N P
	463 Normal		" "	448 N P	446 P 12 days	445 N P	441 P 34 days
	430 Immune		" 5 "	471 N P	457 N P	475 N P	465 N P
	461 Normal		" "	472 P 13 days	458 P 8 days	474 P 12 days	480 N P
IV	428 Immune	Control N P	" P 5 days				
	419 Immune?		Sacrificed 5 days	C 12 N P	C 10 N P	C 8 N P	480 N P
	474 Normal		" 4 "	C 47 P 11 days	C 39 P 5 days	C 45 P 13 days	C 44 N P
	445 Immune		" 3 "	C 43 N P	C 20 N P	C 30 N P	C 23 N P
V	471 Normal	Control N P	" "	C 22 N P	Dead	C 15 N P	C 14 N P
	428 Immune		" P 4 days		C 25 T.B. 11 days		
	C 28 Normal						

N P = No poliomyelitis.

P = Pyleal paralysis poliomyelitis.

† In this monkey, the muscles of the lower legs were almost completely atrophic and the ulnar nerve at the elbow was used for inoculation.

All inoculations were made under ether anesthesia.

appearing in any of the sections from the immune animals, while those from the normal animal C-21, sacrificed 4 days after inoculation, revealed a striking degree of neuronecrosis and degeneration in the lumbar cord with less extensive damage in the cervical enlargement.

In the 4 previously normal animals in which virus was found at various levels in the spinal cord, virus was also recovered from the sciatic nerve in 3 of 8 attempts, once from the site of inoculation, and twice from the nerve proximal to the sciatic notch; in an equal number of attempts, virus was not recovered from the sciatic nerves of any of the immune animals. While virus was found in the spinal cords of 4 of 5 normal monkeys 3 to 5 days after inoculation, it was never found in the spinal cords of immune animals inoculated in the same manner.

These findings raise a question with reference to the interpretation of the results of Howe and Bodian. Perhaps there is no inconsistency since our animals were all infected several times and were solidly immune. The infection was therefore widespread and the immunity was general with no evidence of susceptibility by a different portal of entry, as they describe. Recently, findings somewhat similar to ours have been presented by Habel,¹⁴ who found that rabies virus could be recovered from the brain, cord, and peripheral nerves of normal animals 12 to 25 hours after inoculation into the gastrocnemius muscle, but could not be obtained from nervous tissues of immune animals similarly inoculated.

Lack of monkeys made antibody studies in our series impossible, although it is assumed that neutralizing bodies were present in the blood of most of the immune animals. The experiments offer no definitive information as to the basis of the resistance in the recovered animals, but they do suggest the existence of some mechanism for the arrest of the virus, intimately associated with nervous tissue. Is this possibly due to the removal of some component essential for the proliferation of the virus, or is some inhibiting body present which prevents neuronal entrance?

¹⁴ Habel, K., *U. S. Pub. Health Rep.*, 1941, **56**, 692.

13794

Hydrolysis of Hyaluronic Acid of Human Joint Fluid *in vivo*.CHARLES RAGAN AND ARLENE DE LAMATER. (Introduced by
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There is present in joint fluid a polysaccharide which Meyer¹ had named hyaluronic acid. This polysaccharide is believed to be the substance which is responsible for the increased viscosity of synovial fluid. One may obtain a rough estimate of the amount of polysaccharide present in the joint fluid from the relative viscosity of the fluid. A specific enzyme which hydrolyzes this polysaccharide has been recovered from bacterial filtrates and from various animal organs.² The enzyme has been shown to have many properties similar to the spreading factor of Duran Reynolds.^{3,4} The enzyme has been found to be active *in vitro* and so an attempt was made to determine its activity *in vivo*. In the experiments to be reported here the enzyme was injected directly into the joint space.

Methods. Four patients were taken by chance as they were seen in the clinic or hospital. The enzyme was concentrated by Dr. Karl Meyer, who kindly furnished us with the material. It was derived from bulls' testes. An aqueous solution of the enzyme was made up in small volume. The solution was then passed through a Seitz filter. Fluid was removed from the knee, and the enzyme was immediately injected into the joint space. At varying times thereafter, fluid was again removed from the knee. The joint fluid was cooled, the clot separated by slow centrifugation and the viscosity determined on the supernatant fluid, using an Ostwald viscosimeter under standard conditions. Viscosity values are expressed as multiples of the viscosity of water. The total protein content of the synovial fluid was determined by a specific gravity-gradient method.⁵

Results. As shown in Table I, in all instances in which a potent

¹ Meyer, K., Smyth, E. M., and Dawson, M. H., *J. B. C.*, 1939, **128**, 319.

² Meyer, K., Chaffee, E., Hobby, G. L., and Dawson, M. H., *J. Exp. Med.*, 1941, **73**, 309.

³ Chain, E., and Duthie, E. S., *Nature*, 1939, **144**, 977.

⁴ Hobby, G. L., Dawson, M. H., Meyer, K., and Chaffee, E., *J. Exp. Med.*, 1941, **73**, 109.

⁵ Lowry, O. H., and Hastings, A. B., *J. Biol. Chem.*, 1942, **143**, 257.

TABLE I.

Cause of Hydrarthrosis	Amt of testicular extract injected in water	Time during which enzyme allowed to act, min	Viscosity times water		Protein g %	
			Before enzyme	After enzyme	Before enzyme	After enzyme
Rheumatoid Arthritis	(1) 2.6 mg in 2 cc	16	7.9	4.4	5.0	4.9
	(2) 6.5 mg in 5 cc	130	6.3	2.5	5.0	4.8
Rheumatoid Arthritis	(1) 6.3 mg in 5 cc	170	45.7	32.0*	3.9	3.8
	(2) 4.9 mg in 3 cc	115	119.6	24.2	4.1	4.1
Possible Traumatic Arthritis	(1) 6.3 mg in 5 cc	155	6.6	5.1*	4.9	4.4
	(2) 3.8 mg in 3 cc	90	6.0	1.6	5.1	4.7
Rheumatoid Arthritis	3.6 mg in 3 cc	105	18.0	4.6	5.6	5.4

*Enzyme was also inactive *in vitro*.

enzyme was injected, the viscosity of the joint fluid was markedly reduced. In 2 instances in which an enzyme was injected which had become inactive as measured by *in vitro* hydrolysis no significant reduction in viscosity occurred. Since the change in joint fluid protein was of small magnitude, we believe the changes in viscosity were not the result of dilution caused by adding an aqueous solution.

Another experiment was done to show that the change in viscosity of the joint fluid is brought about by the enzyme itself. This experiment is outlined in Table II. Fluid was withdrawn from the right knee at approximately hourly intervals. After the first hour, 2.5 cc of sterile water was injected into the joint. An hour later there was a small reduction in viscosity of the same order of magnitude as the fall in total protein. At this point a similar amount of water in which 3.4 mg of testicular enzyme had been dissolved was injected into the joint. There was again a slight fall in protein content, but with the addition of the enzyme, the fall in viscosity was marked.

There was no significant change in the underlying disease leading to the hydrarthrosis. There were no marked changes in erythrocyte

TABLE II.

Time	Substance injected into joint space	Joint fluid viscosity times water	Protein g %
8:30		24.3	3.9
9:20		22.8	3.9
9:22	2.5 cc of water		
10:30		19.3	3.7
10:32	3.4 mg of testicular extract in 2.5 cc of water		
11:35		2.1	3.6

sedimentation rate, agglutination with the Group A hemolytic streptococcus, or in the general course of the disease. After a week in most instances, the viscosity of the joint fluid had returned approximately to its original level. There was no change in the tendency of the fluid to reaccumulate in the joint.

13795

Electrophoretic Analysis of Human Semen.*

SEYMOUR GRAY AND CHARLES HUGGINS.

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The seminal proteins are of interest in that they are largely composed of proteoses (60%). By analytical methods globulins account for 21-40% of the total seminal proteins.¹ The present study was undertaken to investigate this nondialyzable portion.

Method. The electrophoretic patterns of normal and pathological semen were studied with the Longworth² modification of the Tiselius³ apparatus. Four pooled normal semen specimens and one specimen from a patient, whose semen contained a persistent thick coagulum, were studied. The semen was centrifuged in a high speed centrifuge at 12,000 revolutions per minute and was then diluted with 1 to 3 volumes of buffer depending on the turbidity of the solution. The solution was then dialyzed for 3 to 4 days at 0° against several liters of the same buffer consisting of 0.025 M veronal, 0.025 N hydrochloric acid and 0.025 N sodium chloride at pH 7.8. The protein solution was then centrifuged at 0° before being introduced into the electrophoretic cell.

The semen was introduced into the 2 lower compartments of the Tiselius cell. The 2 upper compartments of this cell were then disconnected, and buffer was added to the two upper cells. After equilibrium had been established in a constant temperature bath at 0° to 2° the 2 upper cells containing the buffer were made continuous with the 2 lower cells containing the protein and a direct current of 6 milliamperes was applied to the protein solution.

* This investigation was aided by a grant from the National Committee on Maternal Health.

¹ Huggins, C., Scott, W. W., and Heinen, J. H., *Am. J. Physiol.*, 1942, **136**, 467.

² Longworth, L. G., Shedlovsky, T., and Mac Innes, D. A., *J. Exp. Med.*, 1939, **70**, 399.

³ Tiselius, A., *Tr. Faraday Soc.*, 1937, **33**, 524.

TABLE I.
Mobilities of Semen Proteins.

Patient	Mobilities, $U \times 10^5$			
	Albumin	Alpha	Beta	Gamma
WGZ	6.3	4.7	2.2	.6
W	6.0	4.1	2.4	.7
N	6.0	4.4	2.5	.6
LNS	6.0	4.2	2.3	.6
LZ	6.4	4.7	2.3	.6
Normal Serum	6.1	4.3	2.7	.8

All photographs and measurements were made of the protein boundaries descending into the protein solution at the negative pole of the cell.

Results. Four protein fractions are observed electrophoretically in normal and pathological human semen. These 4 proteins have the same mobility as albumin, alpha, beta, and gamma globulin observed in normal serum, and may be presumed to be identical with these protein fractions. (Table I.)

The beta globulin constituted 34.3 to 44.5% of the total protein and was present in largest amounts uniformly in all semen specimens studied. (Table II.) The alpha globulin was the second largest component in all but one semen, and formed 19.8 to 27.8% of the total protein. In the one exception (Table II, case W) the gamma globulin was larger than the alpha globulin, constituting 26.1% of the protein content.

The third largest protein fraction was the albumin which was present in 17.7 to 22.7%. This was observed in 4 of the 5 semen specimens, but in one (Table II, case N) the gamma globulin was larger than the albumin. The smallest protein component in 3 of the 5 cases was the gamma globulin which varied between 11.4 and 21%.

The pathological semen (Table II, case LZ) was identical in protein composition with the normal semens.

Conclusion. 1. Four protein fractions were observed in 4 normal

TABLE II.
Percentage Composition of Semen Proteins.

Patient	Albumin	Alpha	Beta	Gamma
WGZ	17.7	27.8	43.1	11.4
W	22.7	15.9	35.2	26.1
N	18.1	20.5	41.0	20.4
LNS	21.4	23.2	34.3	21.0
LZ (abnormal)	19.0	19.8	44.5	16.7
Normal Serum	64	7	14.5	14.4

and one pathological semen specimens subjected to electrophoretic analysis. 2. The electrophoretic mobilities of these protein fractions after dialysis are identical with those of the albumin, alpha, beta, and gamma globulins of normal serum. 3. Average values for these components as percentages of the total nondialyzable semen protein are: beta globulin, 39.6; alpha globulin, 21.3; albumin, 19.9; and gamma globulin, 19.1. 4. The protein composition of the one pathological semen studied did not differ from the 4 normal semen.

13796

Difference in Response of Certain Strains of Rats to Augmentative Gonadotropic Effect.

JAMES D. HAUSCHILDT AND JOHN S. EVANS. (Introduced by H. Jensen.)

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The phenomenon of synergism between gonadotropic hormones first reported by Evans, Simpson and Austin¹ has been repeatedly confirmed and has since served as an aid in the estimation of the pituitary follicle-stimulating hormone. The most common synergistic combination is that of human chorionic gonadotropin with pituitary follicle-stimulating preparations. It was surprising, therefore, to find that some anterior pituitary preparations prepared in this laboratory and considered fairly certain to contain follicle-stimulating factor in a relatively pure state, gave no augmented response when combined with prolan. The combinations were tested by the ovarian weight increase in normal immature female rats of the Sherman strain obtained from the Rockland Farms. These rats have been used successfully in this laboratory for the assay of either chorionic gonadotropin (prolan) or the gonadotropin of pregnant mare serum alone. They have been found to give responses comparable to those given by Wistar rats from our own colony. However, the elimination of one factor after another finally led to the conclusion that the insensitivity of the strain of rats being used was the only remaining explanation for our failure to get augmentation in test animals when chorionic gonadotropin was combined with a highly purified follicle-stimulating fraction of the sheep pituitary.

When tested on Sprague-Dawley rats, a follicle-stimulating prepa-

¹ Evans, H. M., Simpson, M. E., and Austin, P. R., *J. Exp. Med.*, 1933, **57**, 545.

ration* gave good augmentation when combined with chorionic gonadotropin. A simultaneous and parallel experiment, using Sherman rats brought out forcibly the contrast between the response of each strain. Results are given in Table I. Obviously the ovarian responses of the Sherman rats are simply additive and show no augmentation. This data is merely an example. Using follicle-stimulating preparations of our own we have produced similar results, and have been able to show suitable augmentation in Wistar rats. Results are shown in Table II.

A difference in sensitivity between strains of rats has been noted previously in regard to the anterior pituitary gonadotropins.^{2, 3} When males were used, it was found that Sprague-Dawley rats were many times as sensitive as those of the Long-Evans strain.

TABLE I.
Comparative Responses of Sprague-Dawley and Sherman Rats to the Augmentative Gonadotropic Effect.

Material	Dose	Sherman			Sprague-Dawley		
		Ov. wt mg	Range mg	No. rats	Ov. wt mg	Range mg	No. rats
FSH	4.8 units	25.5	11-67	10	26.8	13-42	10
Prolan	20 I.U.	19.4	14-27	10	28.5	18-40	20
PMS	2.5 I.U.	—	—	—	19	11-27	5
	5 I.U.	—	—	—	22	15-29	10
	10 I.U.	30.8	21-44	8	28	17-36	31
	20 I.U.	70.6	58-94	15	—	—	—
FSH + Prolan	4.8 units						
	20 I.U.	42	14-70	10	112	89-142	10
FSH (4.8 units) + PMS	2.5 I.U.	—	—	—	75	57-85	5
	5 I.U.	—	—	—	89	65-111	10
	10 I.U.	69.9	45-109	8	113	76-135	11
	20 I.U.	79.5	53-109	8	—	—	—
Controls		9.5	9-10	10	15	14.8-16	10

FSH = Follicle Stimulating Hormone.

PMS = Pregnant Mare Serum Hormone.

Prolan = Chorionic Gonadotropin.

1 Unit of FSH = that amount, $\frac{1}{8}$ of which when given subcutaneously once daily for 3 days to hypophysectomized rats (26-28 days old at operation, 7 to 8 days post-operative interval before injections) causes resumption of follicular growth as evidenced at autopsy 72 hours after onset of injections.

* We are greatly indebted to Dr. H. M. Evans for supplying a purified follicle-stimulating preparation through the courtesy of Dr. H. Jensen.

² Fevold, H. L., *Protein Hormones of the Pituitary*, Manuscript of N. Y. Acad. of Sci., 1942.

³ Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., *Endocrinology*, 1940, **27**, 809.

TABLE II.
Comparative Responses of Sprague-Dawley, Sherman and Wistar Rats to the Augmentative Effect.

Material	Dose	Sherman			Wistar			Sprague-Dawley		
		Ov. wt mg	Range mg	No. rats	Ov. wt mg	Range mg	No. rats	Ov. wt mg	Range mg	No. rats
Control		9.5	9-10	7	12.0	10.6-13.7	14	15.0	14.8-16	10
Prolan	20 I.U.	19.3	14-27	10	24.1	18-31	10	28.5	18-40	20
FSH	2 mg	17.9	11-27	10	19.1	15-25	10	18.6	17-21	10
PMS	10 I.U.	24.8	16-34	10	21.9	16-35	10	27.0	20-31	10
	2 mg									
FSH + Prolan	20 I.U.	49.5	36-67	10	73.0	43-112	10	87.0	67-122	10
	2 mg									
FSH + PMS	20 I.U.	55.0	47-67	10	60.0	50-68	5	80.0	55-123	10

FSH = Follicle Stimulating Hormone.

PMS = Pregnant Mare Serum Hormone.

Prolan = Chorionic Gonadotropin.

It is entirely conceivable that a similar difference might be even more pronounced in females even though such a striking difference as we have observed has not been reported as far as we know. It may be that histologic differences between the ovaries of synergistically treated Rockland and Sprague-Dawley rats are not as pronounced as the differences in the gross ovarian appearance and weight. Many assays for the gonadotropic activity of pituitary preparations are conducted on the ovarian weight basis. Since the above results indicate that the ovarian response (weight) depends on the strain of rats employed, results obtained in different laboratories must be compared with care when mixed gonadotropins are assayed.

Summary. A marked difference in the sensitivity of three different strains of rats to the synergistic action of certain gonadotropic hormones has been shown. The three strains, Sprague-Dawley, Wistar and Sherman, give comparable responses to the gonadotropins of pituitary, pregnant mare serum and pregnancy urine when administered singly. Only the Sprague-Dawley and Wistar strains show any augmentative response when purified pituitary follicle-stimulating hormone is given in combination with either chorionic gonadotropin (prolan) or pregnant mare serum hormone.

Augmentation of the Pregnant Mare Serum Gonadotropic Effect.

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It is now generally recognized that the physiological effects observed on administration of the purified gonadotropin from pregnant mare serum into hypophysectomized immature female rats at different dose levels are: Minimal doses produce only repair of the deficient interstitial tissue without any apparent effect either upon the follicles or indirectly upon the vagina and uterus. Larger amounts, in addition to the repair of the interstitial tissue, also cause development of follicles. A further increase in dosage results in the formation of corpora lutea and estrous changes in the vagina and uterus.^{1, 2}

It is at present not possible to decide whether the gonadotropic effect of pregnant mare serum is exerted by two distinct separate principles, one specifically affecting the interstitial tissue of the ovary and the Leydig tissue of the testis, and the second causing follicular growth in the female and germinal tissue development in the male, or whether it is the property of a single individual principle. No pure crystalline gonadotropic pregnant mare serum compound possessing both, or any one of the activities has as yet been isolated. The possibility has to be considered that the two principles (follicle-stimulating and interstitial cell-stimulating) may be linked together in the form of a single composite molecule ("mother molecule") or that they may be attached to the same carrier protein. This theory does not necessarily mean that the two "factors", if combined in a single molecule or if attached to the same carrier protein, are present in the most favorable ratio for complete synergism in the rat, or that the optimum proportions are the same for all species of animals, or for both sexes of any one species.

It seemed of interest to determine whether the gonadotropic response of pregnant mare serum, as tested by the increase of ovarian weight in normal immature female rats, could be augmented either by the addition of a follicle-stimulating or interstitial cell (luteinizing) preparation.

Experimental. The gonadotropic preparations used in this study

¹ Leatham, J. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 590.

² Cole, H. H., Pencharz, R. I., and Goss, H., *Endocrinol.*, 1940, **27**, 548.

were a pregnant mare serum fraction (P.M.S.) assaying 1800 international units per mg, a follicle-stimulating preparation (F.S.H.) obtained from sheep pituitary, containing 40 rat units* per mg, and a chorionic gonadotropin preparation from human pregnancy urine containing 200 international units per mg.

The augmentation experiments were carried out in normal immature female rats (21-23 days old) by subcutaneous administration of the gonadotropic pregnant mare serum preparation alone or of *in vitro* combination of this preparation with either the follicle-stimulating preparation or with chorionic gonadotropin. Injections were made once daily for 3 days, followed by autopsy 24 or 48 hours after the last injection. The animals used in these experiments were either of the Sprague-Dawley or Long-Evans strains. The data given in Table I represent average results obtained with about 20 animals per group (Series 1) and 10 animals per group (Series 2).

From the results recorded in Table I, it is apparent that the addition of a follicle-stimulating anterior pituitary fraction enhances the gonadotropic activity of pregnant mare serum as measured by the increase in ovarian weight. The follicle-stimulating hormone preparation employed in such augmentation experiments naturally has to be comparatively free of the interstitial cell-stimulating or luteinizing principle. As can be seen from Table I, no true augmentative response was observed when pregnant mare serum was administered in combination with chorionic gonadotropin.

TABLE I.
Effect of *in Vitro* Addition of Either Follicle-Stimulating Hormone or Chorionic Gonadotropin to Pregnant Mare Serum Gonadotropin.

Series No.1				Series No.2			
Dosage				Dosage			
P.M.S., I.U.	F.S.H., R.U.	Chorionic Gonadotropin, I.U.	Ovarian wt, mg	P.M.S., I.U.	F.S.H., R.U.	Ovarian wt, mg	
8	—	—	23	5	—	22	
16	—	—	47	10	—	31	
—	4.0	—	22	—	4.8	27	
—	—	20	29	—	3.6	20	
8	4.0	—	80	5	4.8	88	
16	4.0	—	100	10	3.6	117	
8	—	20	35	—	—	—	
16	—	20	62	—	—	—	

Series No. 1 autopsied 24 hours after the last injection. Series No. 2 autopsied 48 hours after the last injection. Uninjected controls ovarian weight 13 mg.

* One rat unit of F.S.H. capable of causing resumption of follicular growth in immature hypophysectomized rats.

The augmentation of pregnant mare serum gonadotropin by the pituitary follicle-stimulating hormone can be considered to be of a specific nature, since it has been shown that non-specific augmenting substances, such as copper or zinc salts, inert proteins, adrenalin, etc., which synergize pituitary extracts, do not augment the hormone from pregnant mare serum.³

Summary. An augmentative effect was observed when pregnant mare serum gonadotropin was administered in combination with a pituitary follicle-stimulating preparation. On the other hand, the addition of chorionic gonadotropin under the same experimental conditions, did not potentiate the gonadotropic effect of the pregnant mare serum preparations.

13798 P

Electrophoretic Analysis of Anti-Papilloma Rabbit Serum.*

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Serum from domestic rabbits bearing the growths of infectious papillomatosis¹ and those injected with extracts of domestic or cottontail rabbit growths or with the purified papilloma virus protein may contain immune bodies demonstrable by complement fixation, neutralization or precipitation.² Recently, studies have been made in this laboratory to learn whether these immunological characters are paralleled by abnormal electrophoretic behavior of such serum.

Sera from individuals of 6 groups of rabbits were examined: (1) domestic rabbits injected intraperitoneally with 0.5 mg of purified papilloma virus protein, once a week for 6 weeks, and serum taken for study every 7 days (for a total of 51 days); (2) domestic rabbits injected intravenously with 0.5 mg of purified papilloma virus protein, twice a week for 6 weeks, and the serum examined after 21 and 50 days; (3) domestic rabbits injected intraperitoneally with

³ Evans, J. S., Hines, L. R., Ceithaml, J. J., and Koch, F. C., *Endocrinol.*, 1940, **26**, 1012.

* This work was aided by the Dorothy Beard Research Fund and by grants from Lederle Laboratories, Inc., Pearl River, New York.

¹ Shope, R. E., *J. Exp. Med.*, 1933, **58**, 607.

² Bryan, W. R., and Beard, J. W., *J. Nat. Cancer Inst.*, 1941, **1**, 607.

1.0 cc of a 7.5% suspension of domestic rabbit warts, twice weekly for 6 weeks; (4) domestic rabbits bearing experimentally-induced growths for 39 to 72 days; (5) domestic rabbits carrying transplanted growths³ in abdominal organs for 115 days; (6) cottontail rabbits bearing experimentally-induced growths for 37 and 40 days. The serum was diluted with 2 volumes of buffer solution of pH 7.8 containing 8.7675 g NaCl, 2.6274 g Na_2HPO_4 , and 0.2071 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ per liter and dialyzed for 4 to 6 days at 2 to 9°C. For electrophoresis the moving boundary method of Tiselius was used and the boundary gradients were recorded as previously described.⁴ The index of serological change was complement-fixing titer.

In the sera of animals receiving the virus intravenously or intraperitoneally, the complement-fixing titer was 1:16 to 1:256. No abnormality either qualitative or quantitative, was seen in the electrophoretic behavior of the sera. The serum from animals receiving suspensions of domestic rabbit growths intraperitoneally fixed complement in 0 to 1:4 dilution, and no change occurred in the electrophoretic pattern.

Definite changes were seen, however, in the sera of animals bearing growths in the skin (complement not fixed in 1 case, but fixed in dilutions of 1:2 to 1:8 in 5 cases) or growths transplanted to abdominal organs (complement fixed in 0 dilution). The electrophoretic diagram of such a serum is seen in Fig. 1. For comparison and to illustrate the significant characters, the diagram of a serum from an animal injected intraperitoneally with papilloma virus protein is given in Fig. 2. The diagram of Fig. 2 shows no difference from that of normal rabbit serum described elsewhere. The significant abnormality of Fig. 1 was the high, relative and absolute, quantity of the complex represented in the beta boundary, both of which were well beyond the limits of variation in normal sera.⁵ The beta complex of Fig. 1 represented 30.3% of the total protein in contrast with 15% as the mean for normal rabbit serum. The significance of the enhanced beta boundary of serum from rabbits carrying warts is not yet apparent. Enhanced beta boundaries have been seen⁶ in human sera from patients with obstructive jaundice,

³ Rous, P., and Beard, J. W., *J. Exp. Med.*, 1934, **60**, 701.

⁴ Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1942, **142**, 193.

⁵ Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *J. Immunol.*, 1942, in press.

⁶ Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A., *J. Exp. Med.*, 1939, **70**, 399.

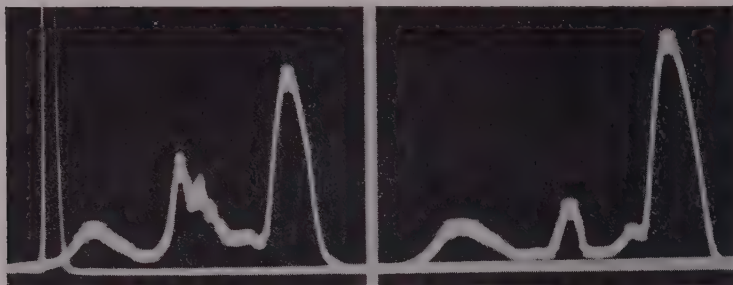


FIG. 1.

FIG. 2.

FIG. 1. Refractive index gradient curve of serum from a domestic rabbit bearing cutaneous papillomas. The curves were photographed at 0 and 276 minutes by the method of crossed slits. The potential gradient was 4.73 volts/cm at 0.2 ionic strength and 1°C.

FIG. 2. Refractive index gradient curve of serum from a domestic rabbit inoculated intraperitoneally with purified papilloma virus. The curve was photographed after 311 minutes' migration. The potential gradient was 4.77 volts/cm at 0.2 ionic strength and 1°C.

multiple myeloma and nephrosis. In these cases portions of the complex responsible for the beta boundary were ether-soluble. Such proved to be the case in the present work since the beta component could be reduced to normal proportions by extraction of the serum with ether. None of the beta component was absorbed by purified virus (9.0 mg virus to 6.0 cc of serum) nor by suspensions of domestic rabbit growths. Only one of 6 sera examined showed no enhancement of the beta boundary and this was from an animal in which retrogression of the growths was in progress. It thus appears that the abnormal component associated with the enhanced beta boundary represents a lipoidal material unrelated to the papilloma antibodies.

In the sera of 2 cottontail rabbits bearing warts, complement-fixing titer 1:16 and 1:32, the findings differed from those with domestic rabbits. In both, change in the beta globulin component was questionable but there appeared to be a definite increase in the gamma globulin. Serological changes after immunization are usually paralleled by changes in the gamma globulin component, but it is noteworthy that the complement-fixing titers of these cottontail rabbit sera were less than those seen in domestic rabbits immunized with purified virus. The lack of increase in the beta boundary may be related to biological phenomena concerned with the growths themselves as reported in an accompanying paper.⁷

⁷ Taylor, A. R., Sharp, D. G., Beard, D., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1942, submitted for publication.

13799 P

Effect of Certain Split Products of Carcinogenic Azo Dyes on Melanin Formation.*

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During the course of some experiments concerned with the production of tumors by the subcutaneous injection of o-aminoazotoluene and 2,2'-azonaphthalene, it was noted that many of the black haired ABC mice developed grey hair following such treatment. The greying was at first local around the site of injection, and later became more general following the pattern described in the literature.¹ Since melanoblast pigmentation is concerned with melanin formation, this observation led to the present investigation which is concerned with the effects of certain oxidation products of these dyes on the tyrosine-tyrosinase reaction. Because there is considerably more information regarding the split products of butter yellow, an azo dye differing from o-aminoazotoluene only in the position of the CH₃ groups, the following compounds were studied: *p*-phenylenediamine, *p*-aminodimethyl aniline, and *p*-aminophenol.² These substances were used because aminoazotoluene and azonaphthalene are insoluble in water. Likewise as a possible oxidation product of azonaphthalene, B-naphthylamine was tested. As a check on our technic, modified after that of Martin, Wisansky and Ansbacher,³ we also tested and confirmed their observations on sulfanilamide, *p*-aminobenzoic acid and hydroquinone. Because of their structural similarity, 3 other compounds were tested, *i. e.*, methyl *p*-phenylenediamine, quinone and *p*-aminoacetanilide.

The technic was briefly as follows: Amounts of tyrosine and of the compound to be studied sufficient to make a 0.001 molar solution of each substance were weighed out and were dissolved in M/15 Sorenson's phosphate buffer at pH 7.0. To 20 cc of this solution was added 0.5 cc of crude tyrosinase obtained from potato juice.

* This investigation was supported by a grant from the Jonathan Bowman Fund for Cancer Research.

¹ Richter, C. P., and Clisby, K. H., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 684.

² Stevenson, E. S., Dobrimer, K., and Rhoads, C. P., *Cancer Research*, 1942, **2**, 160.

³ Martin, G. K., Wisansky, W. A., and Ansbacher, S., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 26.

The latter was prepared by grinding the potato, centrifuging and using the supernatant fluid. The reaction mixture was placed in 125 cc Erlenmeyer flasks and allowed to stand for 18 hours in air, and color observations were made after 12, 6 and 18 hours. Several controls were run with every experiment, *i. e.*, tyrosine plus tyrosinase, tyrosine and compound, compound and tyrosinase, and the compound alone. The end results are briefly summarized in Table I. Only the color and precipitation reactions at the end of the 18 hours are reported because at this time a good precipitate of melanin was invariably produced in the control tube.

Some of the compounds tested are autoxidizable (*p*-aminodimethyl aniline, *p*-phenylenediamine, methyl *p*-phenylenediamine and *p*-aminophenol) and gave similar reactions when added to the tyrosine-tyrosinase solution or when present in the various control runs (Table). Any interpretation of the inhibitory effect of these compounds on this reaction is therefore difficult to make. Sulfanilamide, *p*-aminobenzoic acid, *p*-aminoacetanilide, and *B*-naphthylamine are not autoxidizable and were not oxidized by the enzyme, yet they interfered with the production of melanin from tyrosine. These compounds might conceivably compete with the tyrosine for the enzyme and thus inhibit melanin formation, but this suggestion is by no means certain. Since the non-autoxidizable compounds, which in-

TABLE I.
Effect of Various Compounds on Melanin Formation.
Appearance of solutions after 18 hours.

Compound tested	Compound alone	Compound + Enzyme*	Compound + Tyrosine	Compound + Tyrosine + Enzyme*
Sulfanilamide	Colorless	Colorless	Colorless	Brown-black sol. SL ppt.
<i>p</i> -Aminobenzoic acid	"	"	"	Yellow-brown sol. No ppt.
<i>p</i> -Aminoacetanilide	"	Yellow pink cloudy	"	Red-brown sol. No ppt.
<i>B</i> -Naphthylamine	Pink sol. ppt.	Pink sol.	Pink sol. ppt.	Pink sol. No ppt.
<i>p</i> -Aminodimethyl aniline	Deep royal purple	Purple-black Black ppt.	Purple-black SL ppt.	Purple-black. Black ppt.
<i>p</i> -Phenylenediamine	Red-black	Red-black SL black ppt.	Red-black No ppt.	Red-black. Black ppt.
Methyl <i>p</i> -phenylenediamine	Royal purple Black ppt.†	Red purple Black ppt.‡	Royal purple black ppt.‡	Red-purple. Black ppt.‡
<i>p</i> -Aminophenol	Brown sol. ppt.	Brown sol. ppt.	Brown sol. ppt.	Brown sol. ppt.
Quinone	Orange red†	Deep reddish brown	Orange red	Brown-black. No ppt.
Hydroquinone	Yellow-pink	Deep reddish brown	Yellow-pink	Deep reddish brown. No ppt.
Tyrosine (control)	Colorless	Black ppt.		

* Enzyme = tyrosinase obtained from potato juice.

† Longer time — deep reddish brown.

‡ Observed after 48 hours.

hibit the reaction, show a close chemical relation to the substances which are autoxidizable, (*e. g.*, compare *p*-aminoacetanilide with methyl *p*-phenylenediamine) it is not improbable that the latter compounds may also have an inhibitory effect which is masked by their own color reactions.

The tendency of these autoxidizable substances to form colored compounds and finally a black precipitate may be due to a reaction similar to the formation of quinhydrone from quinone and hydroquinone and of phenoquinone from phenol and quinone as described by Karrer.⁴ The question arises whether or not the formation of melanin could arise in a similar manner. Raper⁵ reports that 3,4-dioxyphenyl alanine is formed upon the oxidation of tyrosine by tyrosinase and that after the oxidation the enzyme can be removed and melanin is still formed. Since the intermediate compound has a quinoid grouping, it seems possible that it might react to form melanin as indicated above.

Summary. The effect of certain split products of some carcinogenic azo dyes and related compounds on melanin formation was studied. It was observed that certain non-autoxidizable compounds such as *p*-aminoacetanilide, *B*-naphthylamine, *p*-aminobenzoic acid and sulfanilamide interfered with this reaction. When chemically related autoxidizable compounds (*p*-aminodimethylaniline, *p*-phenylenediamine, methyl *p*-phenylenediamine and *p*-aminophenol) were used, the color changes in both tyrosine-tyrosinase and control solutions were similar. It is, therefore, difficult to interpret the action of these latter chemicals on melanin formation.

13800 P

Effect of Low Calcium and Vitamin D-Deficient Diet on Bones and Teeth of Mature Rats.*

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Twenty male albino rats which had been raised to an age of 11 months on an adequate stock diet were fed for 5 weeks on a control

⁴ Karrer, Paul, *Organic Chemistry*, New York, 1938.

⁵ Raper, H. S., *Biochemical J.*, 1927, **21**, 89.

* This work was supported by a grant from the Carnegie Corporation.

diet containing 0.462% calcium, 0.458% phosphorus and adequate in vitamin D. Ten of the animals were then transferred to the experimental diet which was deficient in vitamin D and contained 0.007% calcium and 0.450% phosphorus. Calcium and phosphorus balances were determined for each animal over 10-day periods for 220 days. The animals of the control group remained in positive calcium and phosphorus balance throughout the period of observation. The animals fed the deficient diet exhibited a severely negative calcium balance and a positive phosphorus balance.

The humeri and femora and the molar and incisor teeth were dissected free of soft tissue, cleaned, dried and rendered fat-free by continuous extraction with fat solvents. During the removal of the molar teeth from the jaws, it was noted, in the case of experimental rats, that the alveolar bone was soft and friable, the alveolar crest resorbed and the teeth loosened. In the control animals the alveolar bone was well calcified, the crest normal and the teeth firmly fixed in their sockets. The incisor teeth of both groups appeared equally developed and formed. The average weight of the dry fat-free incisor teeth of the two groups was not significantly different. The average percentage of ash in the left femora was not significantly different between the two groups of animals.

The volume and density of the left humerus of each animal was determined, using a specially designed pycnometer and mercury as the suspensum medium. The average volume of the humeri of the experimental animals did not differ from that of the controls but the average density of the bones of the former animals was 10.8% less than that of the controls. The calcium and phosphorus contents of the left humeri, in terms of percent of the dry fat-free weight, were not significantly different between the two groups of animals. However, the calcium and phosphorus contents of these bones in terms of unit volume of bone appeared to be significantly lower in the experimental animals than in the animals fed the adequate diet (Table 1). These results indicate that determinations reported in terms of weight percent do not serve to distinguish osteoporitic from normal bone, but that the degree of osteoporosis can be described in terms of ash weight or mineral content related to unit volume of bone. These results also indicate that mineral and organic phases are proportionally decreased in the osteoporitic bone.

The enamel and dentin of the incisor and molar teeth were separated, using the method of Manly and Hodge.¹ Calcium and phosphorus analyses made on the dentin indicated that no mineral had

¹ Manly, R. S., and Hodge, H. C., *J. Dent. Res.*, 1939, **18**, 133.

TABLE I.
Calcium and Phosphorus Contents of Dentin and Humeri of Rats.

	Molar teeth dentin		Incisor teeth dentin		Humeri	
	Ca %	P %	Ca %	P %	Ca mg/cc	P mg/cc
Controls	30.24 (± 0.60)	13.79 (± 0.79)	26.35 (± 1.08)	14.84 (± 0.61)	301.9 (± 37)	139.5 (± 15)
Experimentals	30.23 (± 0.93)	13.79 (± 0.33)	25.51 (± 0.84)	15.18 (± 0.27)	250.9 (± 17)	115.6 (± 13)

been lost by the molar teeth of the experimental animals. The composition of the dentin of the incisor teeth, which had been completely reformed several times during the period of observation, was not significantly different between the two groups of animals. The numbers enclosed within parenthesis in Table I are the standard deviations of the respective means.

13801

Functional Reorganization Following Preganglionectomy.

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In the human, preganglionic sympathetic outflow affecting the hand is usually over the ventral roots of thoracic nerves 2 through 9.¹ Preganglionic denervation of the upper extremity in cases of Raynaud's disease has been performed frequently by sectioning the 2nd and 3rd spinal nerves, and cutting the sympathetic trunk between the 3rd and 4th thoracic ganglia, leaving T-1 intact to avoid Horner's syndrome.

Evidence has been obtained that in these cases nervous control of vasoconstriction often returns within a relatively short time.² This return has been ascribed to regeneration of the preganglionic fibers.

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¹ Ray, B. S., Hinsey, J. C., Hare, Kendrick, and Geohegan, W. A., *Trans. Am. Neurol. Soc.*, 1942.

² Simmons, H. T., and Sheehan, Donal, *Brit. J. Surg.*, 1939, **27**, 234.

In order to obviate or delay regeneration, section of ventral roots has been proposed as an alternative operation.

Ray¹ reports a case in which the ventral roots of T-2 through T-9 were sectioned, with subsequent evidence of complete sympathetic denervation of the hand. Reflex changes in skin resistance were abolished, and the skin temperature rose to 32°C as compared with 22°C before operation. Within 10 weeks, however, the skin resistance reflex had returned, and the skin temperature had fallen to 24°C. Novocaine block of the ulnar nerve at this time elevated the skin temperature of the 4th and 5th fingers to 29°C. While this return of nervous control may conceivably have been due to regeneration, the shortness of the interval suggests that it may have come about in some other way.

A possible explanation is that the intact preganglionic fibers of T-1 established collaterals within the stellate ganglion and/or middle cervical ganglion, to reinnervate postganglionic neurons which had been deprived of their normal preganglionic supply.

In order to investigate the possibility of such a functional reorganization, an analogous situation was created in cats. In this animal the normal outflow to the forepad is in T-4 through T-10, with an occasional small contribution from T-3.^{3, 4, 5}

If, after this normal outflow were partially or entirely interrupted, it should be found that preganglionic activity to the forepad had developed in T-1 or T-2, or had increased significantly in T-3, it would indicate that such a functional reorganization had taken place.

Method. In 9 cats, under nembutal anesthesia, laminectomy was performed, and 3 to 9 ventral roots were sectioned on one side, within the range T-3 through T-11. Skin temperatures on the normal and operated sides were followed for 2 to 6 months with the Hardy radiometer. At the end of this variable period, the spinal cord was exposed by laminectomy under nembutal anesthesia. After the dura had been opened, the upper thoracic roots were prepared for stimulation by cutting the ventral and dorsal roots close to the cord, securing the distal ends with a fine silk ligature, and cutting the dura about them so that they might be elevated from adjacent tissues. Skin resistance changes were recorded on a 2-channel instrument, each channel consisting of a Wheatstone bridge, a direct-current amplifier, and a magnetically operated stylus. One channel was used

³ Langley, J. N., *J. Physiol.*, 1894, **15**, 176.

⁴ Tower, S. S., and Richter, C. P., *Arch. Neurol. and Psychiat.*, 1931, **26**, 485.

⁵ Gehegan, W. A., Wolf, G. A., Jr., Aidar, O. J., Hare, Kendrick, and Hinsey, J. C., *Am. J. Physiol.*, 1942, **135**, 324.

to record from the stimulated side; the other recorded simultaneously from the contralateral side to make sure that the stimulus was not spreading to the spinal cord, or that stimulation of muscle was not causing reflex changes. A further control on stimulus spread was obtained by observing the pupils. Any pupillary response other than ipsilateral dilatation would have indicated spread of the stimulus. The recording electrodes were small zinc plates applied, over a coating of Cambridge electrode jelly, to the volar surface of the forefoot. The grounded electrode, common to both channels, was fastened to the exposed muscles of the back.

The stimulus was applied to the roots with small bipolar electrodes, and was a 60 cycle sine wave at 2 rms volts measured under load.

This method of stimulation and recording differed in no essential respect from that used previously to determine the normal outflow.³ To insure that minor changes in technic had not altered results, several experiments were carried out on normal animals, and, in several of the operated animals, corresponding roots of the unoperated side were stimulated.

The roots stimulated, as well as those sectioned, were identified at autopsy by dissecting the nerves into the intercostal spaces.

Results. Positive responses from T-1 or T-2 or significantly large responses from T-3 were observed in 6 of the 9 animals. These, together with typical negative records from the normal side of one animal are shown in Fig. 1. In T-1, and to a lesser extent in T-2, there is often an abrupt rise and fall in resistance coincident with the beginning and end of stimulation. This is probably due to muscular movement, and may be distinguished readily from the response due to preganglionic excitation, as the latter has a latent period of about 1 second from the start of stimulation, a more gradual rise which continues after the stimulus, and a much slower return to the base line. The responses shown here have the same form as those previously observed in determining the normal outflow.⁵

Discussion and Conclusions. We conclude from these results that a functional reorganization does take place. Although we cannot be sure that it is due to the actual growth of collaterals, its effect is the same as though this were the case.

Tower and Richter⁴ observed that, following section of the sympathetic trunk below T-3, nervous control of skin resistance returned within a very short time. They suggested that, in some way, roots above this level were able to take over this function, formerly mediated by the roots below. Our results may offer an explanation of their observations, and also, of the fact that preganglionic dener-

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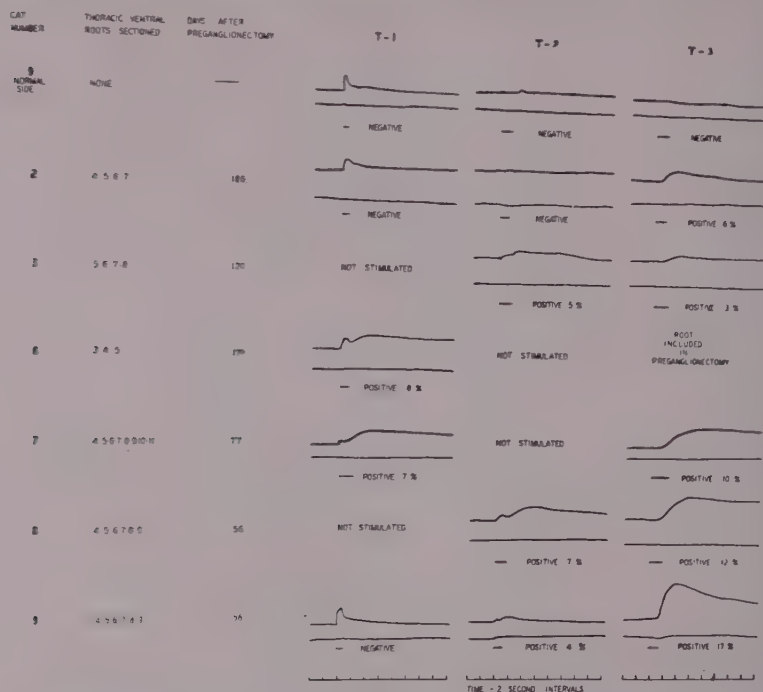


FIG. 1.

Skin resistance changes in response to ventral-root stimulation. In each record, the stimulated side is on the upper line; the contralateral side on the lower line. The short horizontal lines indicate the period of stimulation. Changes are indicated as a percentage of the basal resistance. The basal resistance varied from 6,000 to 12,000 ohms. The top and bottom sets of records are, respectively, responses to stimulation of the normal and operated sides of the same animal.

vation of the lower extremities has been generally more successful than that of the upper extremities.

It is known that return of sympathetic innervation may be brought about by actual regeneration of the preganglionic fibers.⁶ Functional reorganization must also be considered in planning operations upon the sympathetic system.

Summary. Functional reorganization in the sympathetic nervous system following preganglionectomy has been demonstrated, using the cat as the experimental animal. Skin resistance change in the forepad, in response to stimulation of the ventral roots, was used as an indicator of preganglionic activity. Following interruption of preganglionic pathways to the forepad it was found that preganglionic

⁶ Hinsey, Joseph C., Phillips, Robert A., and Hare, Kendrick, *Proc. Am. Physiol. Soc.*, 1939, **126**, 534.

pathways develop from roots which normally contribute nothing to the forepad.

This may offer additional explanation for early relapse following preganglionic section for Raynaud's disease in the hands, and for the fact that operations for this disease are generally more successful in the feet than in the hands.

13802

Quantitative Studies on Tumor Production in Mice by Benzpyrene.

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From the Department of Pathology, University of Liège, Belgium.

Quantitative studies on the response of animals to benzpyrene do not extend to the smallest effective doses; precise data on the effect of small amounts of other carcinogens are likewise scarce.

Brock, Duckrey and Hamperl¹ inserted a collodion sac containing crystals of 3-4 benzpyrene (500 to 25,000 γ) into the peritoneum of rats. They estimated that about 1 γ of benzpyrene passed the collodion membrane daily; this was sufficient to induce sarcoma in the majority of animals. Oberling and M. and P. Guérin² obtained sarcomata in 4 of 35 rats that had received a single subcutaneous injection of 100 γ of benzpyrene in olive oil and in 1 of 10 rats injected with 50 γ of benzpyrene. Shimkin and Andervont³ injected male mice of C₃H stock with 0.2 cc of tricapylin containing from 250 to 2000 γ of benzpyrene. All animals developed tumors after injection of 2000 and 1000 γ ; 19 of 20 and 18 of 20, respectively, were positive after injection of 500 γ and 250 γ . The same amounts of benzpyrene were injected into hybrid mice, but the results were more irregular, and tumors failed to appear in at least one-third of the injected animals. Bryan and Shimkin,⁴ analyzing the values of latent period of tumors obtained by the former authors in C₃H mice (87 to

* Graduate Fellow of the Belgian American Educational Foundation.

† These experiments were performed in the Department of Pathology of the University of Liège from November, 1938, to May, 1940.

¹ Brock, N., Duckrey, H., and Hamperl, H., *Arch. f. exp. Path. u. Pharmacol.*, 1938, **180**, 709; *Arch. f. klin. Chirurg.*, 1938, **194**, 250.

² Oberling, Ch., Guérin, M. and P., *Bull. Assn. Fr. Etude Canc.*, 1939, **28**, 198.

³ Shimkin, M. B., and Andervont, H. B., *J. Nat. Canc. Inst.*, 1940, **1**, 57.

⁴ Bryan, W. R., and Shimkin, M. B., *J. Nat. Canc. Inst.*, 1941, **1**, 807.

108 days), found a straight line relationship between average latent period and the logarithm of the injected dose, excluding the 2000 γ benzpyrene values, as no further enhancement of activity was obtained above 1000 γ . This relationship was confirmed by reliable data published by other investigators^{5, 6} on the latent period of tumors induced by 1-2-5-6 dibenzanthracene. Smaller amounts of this carcinogen have also been tested. Two of 167 mice injected by Dobrovolskaia-Zavadskaia⁵ with 2.5 γ of dibenzanthracene developed sarcomata; whereas 1.25 γ failed to induce tumors in 158 mice. Lettinga⁶ obtained tumors in 4 of 20 mice after subcutaneous injection of 12.5 γ of dibenzanthracene (divided into 5 injections), but failed in 20 mice after injection of 5 γ . Shear^{7, 8} produced a sarcoma in 1 of 18 strain A mice 13 months after subcutaneous insertion of a 0.001% dibenzanthracene-cholesterol pellet containing 0.4 γ of the carcinogen, but failed in 15 and 18 mice with 0.1% and 0.01% pellets. The minimum concentrations of the carcinogen in the cholesterol pellets with which Shear and Ilfeld⁹ produced sarcomata in strain D mice were 1% of dibenzanthracene and of methylcholanthrene (70 to 600 γ) and 5% of benzpyrene (350 to 3000 γ). Oberling, Sannié and P. and M. Guérin¹⁰ obtained local sarcomata in rats after intracerebral application of 1 drop of 1:1000 methylcholanthrene solution in oil.

Experimental. The mice used were of an albino stock kept at the University of Liège. Eight groups of 20 mice received a single injection under the skin of the abdomen of 3-4 benzpyrene[†] dissolved in 0.5 cc of neutral olive oil. The dose per mouse in each group varied by geometric progression, as shown in Table I, from 4×10^3 to 4×10^{-2} γ . The oil alone produced no tumors on repeated injection into 15 mice.

Table I indicates that 4 γ are the smallest amount of benzpyrene which produced a tumor when administered in a single dose subcutaneously in 0.5 cc of olive oil. Four-tenths γ and .04 γ failed to produce sarcomata at the site of injection. The two carcinomata of the breast observed were probably spontaneous. The percent of tumors obtained was larger when larger amounts of the chemical

⁵ Dobrovolskaia-Zavadskaia, N., *C. E. Soc. Biol.*, 1938, **120**, 1055.

⁶ Lettinga, T. W., *De Carcinogene Werking van kleine Doses 1-2-5-6 Dibenzanthraceen*, Academisch Proefschrift, Amsterdam; Van Gorcum and Co., Assen, 1937.

⁷ Shear, M. J., *Am. J. Canc.*, 1936, **26**, 322.

⁸ Shear, M. J., and Lorenz, E., *Am. J. Canc.*, 1939, **36**, 201.

⁹ Shear, M. J., and Ilfeld, F. W., *Am. J. Path.*, 1940, **16**, 287.

¹⁰ Oberling, Ch., Sannié, Ch., Guérin, P. and M., *C. E. Soc. Biol.*, 1939, **131**, 455.

[†] Obtained from Meurice, Union Chimique Belge.

TABLE I.
Results of Subcutaneous Injection of Diminishing Amounts of Benzpyrene.

Amt in γ	No. of mice*	No. of tumors†	% tumors	Avg latent period (days)	Avg survival tumor-bearing mice (days)‡
4000	9	6	66.6	112	130
1265	10	7	70	122	154
400	5	1	20	145	178
126.5	12	8	66.6	155	199
40	5	1	20	122	215
4	9	1	11.1	187	260
0.4	15	0 (2§)		(226)	(255)
0.04	9	0 (1§)		(245)	(294)

*Alive at the appearance of the first tumor. A large number died early because of fighting; some were killed for histological examination.

†Sarcomata, occasionally with epithelioma.

‡The mice were allowed to die. Ten mice were killed when bearing large tumors. Three had been injected with 4000 γ , 3 with 1265 γ , 1 with 126.5 γ , 1 with 40 γ , 1 with 4 γ and 1 with 0.04 γ . In calculating the average length of survival, the day of death of these animals was considered to be the tenth day after they had been killed.

§Adenocarcinoma of breast.

were injected. A discordant value (400 γ group) can be explained by the small number of animals used; moreover, the mice were not of an inbred stock. On the whole, these data confirm and extend those of Shimkin and Andervont.³

There is a definite trend toward lengthening of the latent period with the decrease of the amount injected. The values obtained between 126.5 and 4000 γ fit a linear relation between the average latent period and the logarithm of injected dose, and the slope is similar to that derived from the values of Shimkin and Andervont.^{3, 4} Whether this relation applies also to doses of benzpyrene below 126.5 γ cannot be stated because of the small number of tumors produced.

The tumors were regularly preceded by induration around the oil cyst, but carcinogenesis did not always follow the induration. Microscopically, areas of fibrosis and chronic inflammation surrounded the oil cyst. In a few cases small groups of seemingly malignant cells were seen about the oil cyst; these animals were reported as negative, as the onset of a tumor was dated arbitrarily from the appearance of a nodule measuring approximately 1 mm across.

The average survival time of tumor-bearing mice is shortened in a linear relation with the logarithm of the injected dose. Shimkin¹¹ has already shown that the mortality curve parallels the curve of appearance of tumors, despite the unpredictable rate of tumor growth.

In order to study the *rôle of volume* in carcinogenesis, a constant

¹¹ Shimkin, M. B., *J. Nat. Canc. Inst.*, 1941, **1**, 761.

TABLE II.
Results of Injection of 400 γ of Benzpyrene in Varying Amounts of Oil.

Vol. oil (cc)	No. inj. mice	No. of tumors*	Avg latent period (days)
1	20	8	154
0.5	20	10	107
0.25	20	9	103
0.125	20	11	144
0.0625	20	4	128
0.03	20	5	133
0.015†	20	2	150
Total	140	49	128

*Sarcomata—Results read on the 204th day.

†Soluble at 40°C.

amount of benzpyrene (400 γ) was dissolved in olive oil varying in amount from .015 cc to 1 cc and injected in groups of 20 mice each.

When the solvent was .125 cc or more, at least 40% of tumors were produced. When the solvent was .0625 cc or less, the percent of tumors produced was below 25. The corresponding average latent periods were, however, without relation to the volume injected. These data may perhaps be explained by assuming that if a concentrated solution is injected, a relatively smaller number of cells is exposed to the irritant, or that the carcinogen is more quickly eliminated, or that the tissue derangement, which is believed to favor initiation of tumor growth, is less.¹²

The *relationship between the injected oil and tumor* or inflammatory reaction was studied systematically. The autopsies were performed under ultraviolet light, using a mercury lamp with Wood's filter. After shaving the skin, the presence of a very strong fluorescence often enabled the localization of an oil cyst beneath the skin, and minute amounts of fluorescent material could be identified in the tissues. This procedure guided the selection of areas for microscopic study. In mice injected with 4 γ or less fluorescence was not sufficient for the localization of the carcinogen.

In order to study the rate of absorption of the carcinogenic oil, the oil cysts were punctured with a thin needle and washed from 3 to 5 times with spectrographically pure petroleum ether until the fluorescence disappeared, but traces of fluorescent product could sometimes not be washed out of the wall of the cyst. The oil and petroleum ether mixture was evaporated in a vacuum and in the dark to a constant weight. The samples were kept in the ice box and in the dark to avoid disappearance of the fluorescence and of the ab-

¹² Dunning, W. F., Curtis, M. R., and Wood, F. C., *Am. J. Canc.*, 1940, **39**, 70.

sorption spectrum characteristic of benzpyrene. The uninjected solutions of benzpyrene in oil were not as labile. The material dissolved in petroleum ether was tested by absorption-spectrography under variable thickness (25 cc cups). The technic used allowed a rapid determination of amounts of 3-4 benzpyrene above 1 γ ; the precision was about $\pm 5\%$. The amount of 3-4 benzpyrene identified was lower than expected from the weight of the remaining oil. The results also show that the rate of resorption of the oil was irregular and without relation to the amount of benzpyrene injected or to the appearance of a tumor. These experiments will be more fully described when the data are obtainable from Belgium.

Thus, despite the variability of local factors, such as the encapsulation and the rate of resorption of the carcinogenic oil, despite the irregular rate of growth of the neoplastic elements, definite relationships can be found between the amount of carcinogen used and the number of induced tumors, their latent period, or the average survival of tumor-bearing mice.

Summary. Amounts of benzpyrene ranging between .04 γ and 4 mg dissolved in 0.5 cc of olive oil were injected subcutaneously into mice. The smallest amount producing a sarcoma was 4 γ . The data suggest a linear relationship between the logarithm of the injected dose and the average latent period of tumors, as found by Bryan and Shimkin. A similar relationship was found between dose and average length of survival of the tumor-bearing animals. When dissolved in .125 to 1 cc of oil, 400 γ of benzpyrene produced tumors in 40% or more of the mice. When dissolved in from .0625 to .015 cc of oil, the incidence of tumor production was 25% or less. No relationship was found between average latent period and volume of injection. A technic is described for the study of the rate of resorption of benzpyrene and oil.

I am indebted to Dr. Jean Firket, Department of Pathology, University of Liège, for his kind advice; to the late Mr. Victor Henri and to Mr. Michel Herquet, Department of Physical Chemistry, University of Liège, for their coöperation in the spectrographical analyses.

Yeast-Growth-Promoting Effect of Diaminocarboxylic Acid Derived from Biotin.*

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York City.*

In a previous report in which it was shown that biotin is a cyclic urea derivative, the question was raised whether the urea structure is of significance in the affinity of biotin for avidin.¹ Some evidence in favor of this possibility is afforded by the results we have obtained in a study of the yeast-growth-stimulating activity, both in the presence and absence of avidin, of the diaminocarboxylic acid derived from biotin. In the formation of the diaminocarboxylic acid the urea ring of biotin is hydrolyzed with strong Ba(OH)₂. Under these conditions the rest of the molecule remains intact as demonstrated by the fact that treatment of the diaminocarboxylic acid with phosgene yields biotin identical with the original starting material.¹

The yeast-growth-stimulating activity of the diaminocarboxylic acid sulfate prepared as previously described² was determined by the assay method described by Snell, Eakin and Williams³ with minor modifications. On a molecular basis the carefully purified diaminocarboxylic acid sulfate was found to have approximately 10% of the activity of biotin. The activities of the two compounds were compared at the levels which produced half-maximum growth. Repeated recrystallization of the diaminocarboxylic acid sulfate did not change its activity.

The activity of the diaminocarboxylic acid may be due either to an intrinsic activity of the molecule or to the conversion of the compound to biotin within the yeast cell. Since the full yeast-growth activity and optical rotation of biotin can be restored by again forming the urea ring by treatment of the diaminocarboxylic acid with phosgene, the lower yeast-growth activity of the diaminocarboxylic

* The authors wish to express their appreciation to the S.M.A. Corporation for a research grant which has aided greatly in this work.

¹ Melville, D. B., Hofmann, K., and du Vigneaud, V., *Science*, 1941, **94**, 308.

² Hofmann, K., Melville, D. B., and du Vigneaud, V., *J. Biol. Chem.*, 1941, **141**, 207.

³ Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1940, **62**, 175.

acid is not due to racemization in the preparation of this compound, but must be due to the absence of the urea ring. This strongly suggests the importance of the urea ring to the high degree of growth-stimulating activity possessed by biotin.

Although avidin can completely inhibit the activity of biotin, it was found that it does not affect the activity of the diaminocarboxylic acid. The purified avidin was prepared by the method of Eakin, Snell and Williams.⁴ 0.35 γ of our avidin concentrate was required to completely inhibit the amount of biotin, 7×10^{-4} γ , which produced half-maximum growth of the yeast culture. The same amount of avidin had no effect on the activity of the diaminocarboxylic acid. Increasing amounts up to 100 γ of our avidin preparation did not affect the growth-stimulating properties of the diaminocarboxylic acid.

The lack of inhibition of the diaminocarboxylic acid by avidin demonstrates that the activity of the diaminocarboxylic acid could not be due to the presence of any unchanged biotin. These results lead to the inference that the urea ring is vital for the effect of avidin on biotin; for under the same conditions, as soon as the urea ring is opened, avidin does not affect the growth-stimulating property of the new compound. If for its yeast-growth-stimulating action the diaminocarboxylic acid is converted to biotin, this conversion evidently takes place within the cell where the avidin cannot counteract the biotin as it is formed.

Summary. The diaminocarboxylic acid resulting from the hydrolysis of the urea ring of biotin is capable of stimulating the growth of yeast in a biotin-free medium. The compound possesses about 10% of the activity of biotin. The yeast-growth-promoting activity of the diaminocarboxylic acid is not inhibited by avidin.

The authors wish to express their appreciation to Miss Eleanor Hague and Miss Jean Steigerwalt of this laboratory for carrying out the assays.

⁴ Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1941, **140**, 535.

Evaluation of Germicides with Relation to Tissue Toxicity.

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The consensus of opinion of investigators and clinicians is that tissue toxicity of germicides should be evaluated as well as their germicidal power. Tissue culture,¹⁻⁴ manometric,^{5, 6} and phagocytosis inhibition^{7, 8, 9} studies have been made. The skin and blood, the primary barriers to infection, are tissues most affected by the use of germicides. From a clinical standpoint, therefore, the criterion of tissue toxicity should be based on the destruction of these two tissues. Nyé,⁷ Welch and Hunter,⁸ and Welch and Brewer⁹ tested germicides, using inhibition of phagocytosis as the criterion of toxicity.

The present study was concerned with an attempt to standardize certain factors in the phagocytosis inhibition technic with regard to time relationships and the quality and quantity of organic matter entering into the reaction. A test organism was used which needed no artificial opsonization and was comparatively resistant to the action of germicides in general. An attempt was also made to establish the relative sensitivity of skin and blood to the destructive effects of germicides.

The so-called Toxicity Index has been described as toxicity end point/germicidal end point.⁴⁻⁹ This same ratio was used in the present study. The toxicity end point was the dilution of antiseptic completely inhibiting phagocytosis. The germicidal end point was the dilution completely killing the test organism in the presence of blood.

* Portion of thesis submitted in partial fulfillment of the requirements for the degree Master of Science in the Graduate School of the University of Illinois.

¹ Lambert, R. A., and Meyer, J. R., *Proc. Soc. Exp. Biol. and Med.*, 1926, **23**, 429.

² German, W. M., *Arch. Surg.*, 1928, **18**, 1920.

³ Buchsbaum, R., and Bloom, W., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 1060.

⁴ Salle, A. I., McOmie, W. A., Schechtmeister, I. L., and Foord, D. C., *Proc. Soc. Exp. Biol. and Med.*, 1938, **37**, 694.

⁵ Bronfenbrenner, J., Hershey, A. D., and Doubly, J. A., *J. Bact.*, 1939, **37**, 583.

⁶ Ely, J. D., *J. Bact.*, 1939, **38**, 391.

⁷ Nyé, R. N., *J. A. M. A.*, 1937, **108**, 280.

⁸ Welch, H., and Hunter, A. C., *Am. J. Pub. Health*, 1940, **30**, 129.

⁹ Welch, H., and Brewer, C. M., *J. Immunol.*, 1942, **43**, 25.

The toxicity end point was determined as follows: Four-tenths ml of decreasing concentrations of the germicide (diluted with 0.85% NaCl) was placed into a series of 1 x 10 cm test tubes in a water bath at 37°C. Four-tenths ml fresh human whole blood, containing 0.25% sodium citrate, was added and the final dilution of the germicide was calculated. After 30 minutes' incubation at 37°C, during which time the tubes were frequently shaken, 0.2 ml of a standardized suspension of the test organism was added. The bacterial suspension consisted of 240-270 million organisms per ml in 0.85% NaCl, from a washed 24-hour nutrient agar culture of a phagocytatable strain of *Staphylococcus albus*, isolated from the air and found to ferment mannitol but not to coagulate plasma. After addition of the bacteria, the contents of the tubes were mixed at 37°C in a device which simultaneously rotated the tubes and tipped them end to end for a period of 5-10 minutes in order to assure intimate contact between leukocytes and bacteria. A smear from each tube was stained with Wright's stain. After examination of 25 polymorphonuclear leukocytes, the slide, representing the highest dilution of the germicide, which shows an average of less than one engulfed bacterium per cell was considered as the toxicity end point. Controls with 0.85% NaCl in place of the germicide were used to show complete phagocytosis under the test conditions.

The germicidal end point was determined by placing 0.4 ml of decreasing concentrations of the germicide in a series of 1 x 10 cm sterile test tubes at 37°C to which were added 0.4 ml of citrated human blood (old enough to contain no living leukocytes) and 0.2 ml of the bacterial suspension as described above. The final dilution of the germicide was noted. After 30 minutes' incubation at 37°C, one 4 mm loopful from each tube was transferred to broth. A second transfer of four 4 mm loopfuls was made to avoid bacteriostatic effects. The transfers were incubated at 37°C for 48 hours. The highest dilution of the germicide showing no growth was taken as the germicidal end point. Positive controls using bacteria and blood, and sterile controls consisting of blood and saline were used.

The results are summarized in Table I. A toxicity index of less than one indicates a germicide, whose lowest germicidal concentration is non-toxic. On the other hand a value of more than one indicates relative increased toxicity of the chemical to phagocytosis. Four compounds (in aqueous solution only) display toxicity indices of less than one, with efficiency ranges as follows: mercuric chloride, 1:1030-1:1730; N-N'-dichloroazocarbonamide, 1:3000-1:5000; alkyl-dimethyl-benzyl-ammonium chloride, 1:3370-1:3970; and 4 ni-

TABLE I.
Order of Efficiency of 24 Germicides as Measured by the Toxicity Index.

	T.	G.	T.I.
1. Mercuric chloride	1:1030	1:1730	0.59
2. N-N1-dichloroazocarbonamide	1:3000	1:5000	0.60
3. Alkyl-dimethyl-benzyl-ammonium chloride (aqueous)	1:3370	1:3970	0.85
4. 4-nitro-5-hydroxymercuri-o-cresol (aqueous)	1:1330	1:1400	0.95
5. Phenyl mercuric nitrate	1:2500*	1:2100*	1.2
6. 2-furylmercuric hydroxide (aqueous)	1:5700*	1:3700*	1.5
7. 2-furylmercuric hydroxide (tincture)	1:8000	1:5000	1.6
8. Phenol	1:100	1:63	1.6
9. Iodine (aqueous)	1:200	1:120	1.7
10. 4-nitro-5-hydroxymercuri-o-cresol (tincture)	1:3400	1:1570	2.2
11. Iodine (tincture)	1:470	1:180	2.6
12. Alkyl-dimethyl-benzyl-ammonium chloride (tincture)	1:19,000	1:7000	2.7
13. 1,3 hydroxy-4-hexylbenzene	1:4000	1:1400*	2.8
14. Tinc. cresol and mercuric chloride	1:12	1:4	3.0
15. Tri-cresol	1:800	1:200	4.0
16. 50% alcohol—10% acetone	1:13	1:3.3	4.0
17. Alcohol 95%	1:17	1:3.7	Av. 5.2
18. Alcohol 70%	1:27	1:4.7	
19. Sodium-ethyl-mercurithiosalicylate (tincture)	1:17,700	1:2300	7.7
20. Disodium 2,7 dibromo-4-hydroximercuri-fluorescein (aqueous)	1:630	1:55*	11.5
21. Disodium 2,7, dibromo-4-hydroximercuri-fluorescein (tincture)	1:5300	1:430	12.5
22. Sodium-ethyl-mercurithiosalicylate (aqueous)	1:1470	1:100	14.7
23. Sodium hypochlorite	1:20	1:1*	20
24. Tincture Green Soap	1:1830	1:2	900

*An excess of germicide in original concentration was required to obtain these results.

T = Toxic End Point. Highest dilution completely inhibiting phagocytosis.

G = Germicidal End Point. Highest dilution completely sterilizing infected blood.

T.I. = Toxicity Index = T/G.

tro-5-hydroxymercuri-o-cresol, 1:1330-1:1400. The efficiency range is regarded as the limits of concentration within which a germicide, under the conditions of the test, will completely destroy bacteria but will not destroy tissue. A substance with a toxicity index greater than one has no such efficiency range.

Further perusal of Table I indicates that the chlorine compounds rate the highest while the organic mercurials rate among the poorest, although this is not a strict rule. The phenolic compounds hold a more or less intermediate position.

No tincture preparation shows a toxicity index of less than one in this study. This would seem to indicate that the solvent itself is more toxic to phagocytosis than to bacteria. This is borne out by the results for the common tincture solvents, 50% alcohol-10% acetone and 70% alcohol.

Guinea pigs were used to determine the relative sensitivity of skin

and blood to the action of germicides. Areas of 1 sq cm on the shaved skin were scarified. The various germicides were applied constantly (to prevent evaporation) to cotton compresses on the scarified areas. Saline solution was used on control areas. Intra-peritoneal injections of 1 ml of 15% urethane per 100 g of body weight were used to keep the animals under constant anesthesia. Results show that of all germicides tested the highest dilution, previously found to completely inhibit phagocytosis, manifested no toxic reaction to scarified skin for equal periods of exposure.

Discussion. A method standardizing certain factors in the phagocytosis inhibition technic of rating germicides is proposed. In previous studies^{4, 8, 9} the germicidal power was measured by incubating a mixture of germicide, blood, and bacteria for 30 minutes. The same principle was applied in the present study. The toxic power in the previous studies was determined by 30 minutes' incubation of a mixture of germicide, fresh blood, and opsonized bacteria. This in reality measured the instantaneous inhibition of phagocytosis. The present study, however, measured toxicity by first incubating the germicides and fresh blood for 30 minutes and then adding the bacteria. This standardized the time relationships of the toxicity and germicidal tests.

The previous studies made use of an artificially opsonized saline suspension of the antigen for the toxicity tests and a broth-culture suspension for the germicidal tests. In the present study the test organism, which need not be artificially opsonized, was used in saline suspension for both the toxicity and the germicidal tests. This standardized the amount of organic matter entering into the reaction.

Summary. Application of the technic described, considering tissue toxicity as well as germicidal power, indicates that in general the chlorine compounds rate the highest, phenolic compounds next, and organic mercurials rate the lowest as efficient germicides. This corroborates certain previous studies.^{4, 8, 9} Tincture preparations in general are not ideal germicides since the solvents are more toxic to phagocytosis than to bacteria.

The toxicity and germicidal end points found in this study indicate that a large number of germicides in general use are too concentrated. Germicides should be utilized in concentrations which are germicidal but non-toxic to tissue. The efficiency range is defined.

Of the natural barriers to infection, *i. e.*, skin and blood, the blood is more sensitive to the destructive action of germicides. Therefore the phagocytosis inhibition technic may be regarded as a valuable aid in the evaluation of germicides for clinical use.

SECRETARY'S REPORT

April 1, 1941-April 1, 1942

The annual meetings of the Council, the Board of Editors, the Membership Committee and the Section Secretaries were held in Boston April 2 and 3, 1942.

Editorial Matters. To further raise the standards of the PROCEEDINGS the Council voted progressively to eliminate "trivial" manuscripts. In the rare instances of multiple publication, the author is to be notified at the first offense, and at the second informed that no further manuscripts will be accepted from him.

When a manuscript is received, the author-member or sponsor is immediately notified. When a group of manuscripts is received from a Section Secretary, acknowledgment is sent to the Secretary. Absence of such acknowledgment means manuscript has been lost in transmission.

There were several violations of our copyright. To reduce this type of misunderstanding, a notice of copyright is printed on each reprint.

In conformity with the policy of rotation of editors after five years' service, Doctors Danforth, Hooker and Ivy retire. Dr. Wilson asked to be relieved. The Society is greatly indebted to these editors for extremely conscientious and excellent service. The following editors were elected: Doctor F. D. W. Lukens, Philadelphia; Dr. J. Bronfenbrenner, St. Louis; Dr. M. B. Visscher, Minneapolis, and Dr. C. L. A. Schmidt, San Francisco.

Beginning October 1942, the Proceedings will be printed in two columns a page. Authors are therefore requested to prepare tables and illustrations to be printed in, or across, two columns, and to indicate which format is preferred.

To avoid delay members are requested to note the changes in "Instructions to Authors" on cover of PROCEEDINGS beginning next October.

Finances. With the many incalculable factors arising out of the war crisis, it is pleasant to report a surplus for the fiscal year of \$3167. Though the crisis continues, and budget making extremely difficult, the Council deemed it wise to make the following changes:

1. Reduce annual membership dues for the coming fiscal year from \$4.50 to \$4.00.
2. Reduce charge for excess space from 50% to 40% of cost.
3. Reduce charge for illustrations and tables from 50% to 40% of cost.

4. Members in war zones, who are in arrears for dues, may have these arrears cancelled for the years September 1940 to September 1942.

5. Libraries in war zones that have discontinued their subscriptions may receive the PROCEEDINGS, or have them held here for shipment, for the next fiscal year, without payment. The number of such subscriptions shall not exceed ten.

Membership. For reasons given in the previous report of the Secretary, applications for membership should be received by December 15. One hundred and eleven applications were approved, also 18 applications from South America. Fifteen applications were referred to the Membership Committee for reconsideration. Twelve applications were deferred pending further publication in experimental biology or medicine. Fourteen applications were received too late for adequate consideration. The Council reiterated its policy that applicants to active membership shall not be elected by each Section but only by the Council upon recommendation of the National Membership Committee.

The following were elected emeritus members: R. H. Chittenden, F. Ramaley, E. G. Hastings, H. A. McGuigan, A. F. Coca, and J. C. Torrey.

The following resignations were accepted with regret: Doctors K. S. Bishop, N. F. Blau, S. Bliss, A. Chace, M. Cohen, E. C. Cole, M. Collett, H. A. Davenport, R. Goldschmidt, H. Heft, F. M. Huntoon, V. C. Jacobson, G. H. Maughan, G. L. Muller, H. E. Pearse, L. B. Pett, L. B. Richards, E. L. Scott, W. J. Scott, E. B. Sundstroem, J. P. Visscher.

Delays or misunderstandings. 1. Applications for membership should be in the General Secretary's office by December 15, should include full titles of each publication, have three endorsements, be approved by Section Membership Committee. 2. Applications from workers south of the United States may be endorsed by members in the United States, may be approved by Section Committee, or by the special committee, viz., Doctors Fulton, Gasser, Lambert and Leake.

Delays in publication have been due principally to (a) absence of letter from sponsor, (b) no statement whether manuscript is preliminary or complete, (3) hasty preparation of manuscript, (4) received too late for current number, (5) tables contain no legend, symbols not understandable, or not arranged to be printed vertically on page.

Acknowledgments. The Society is greatly indebted for continued and excellent service to

Dr. Emil Baumann (member) for painstaking and most excellent indices of the PROCEEDINGS.

Mr. H. G. Friedman (non-member) for ever watchful and expert advice on investments of the Society.

Mr. Leon Leighton (non-member) for continued advice on the legal phases connected with mortgage investments of the Society.

PAST OFFICERS

<i>Date</i>	<i>President</i>	<i>Vice-President</i>	<i>Secretary</i>	<i>Treasurer</i>
1903-04	S. J. Meltzer	W. H. Park	W. J. Gies	G. N. Calkins
1904-05	S. J. Meltzer	J. Ewing	" "	" "
1905-06	E. B. Wilson	E. K. Dunham	" "	" "
1906-07	S. Flexner	E. K. Dunham	" "	" "
1907-08	S. Flexner	T. H. Morgan	" "	" "
1908-09	F. S. Lee	T. H. Morgan	" "	G. Lusk
1909-10	F. S. Lee	W. J. Gies	E. L. Opie	" "
1910-11	T. H. Morgan	W. J. Gies	" "	" "
1911-12	T. H. Morgan	P. A. Levene	G. B. Wallace	" "
1912-13	J. Ewing	P. A. Levene	" "	C. Norris
1913-14	J. Ewing	C. W. Field	H. C. Jackson	" "
1914-15	G. Lusk	W. J. Gies	" "	J. R. Murlin
1915-16	G. Lusk	G. N. Calkins	H. C. Jackson	" "
1916-17	J. Loeb	W. J. Gies	" "	" "
1917-19	W. J. Gies	J. Auer	" "	" "
1919-21	G. N. Calkins	G. B. Wallace	" "	" "
1921-23	G. B. Wallace	J. W. Jobling	" "	" "
1923-24	H. C. Jackson	J. W. Jobling	V. C. Myers	" "
1924-25	H. C. Jackson	J. W. Jobling	A. J. Goldforb	" "
1925-27	J. W. Jobling	S. R. Benedict	" "	" "
1927-29	S. R. Benedict	P. Rous	" "	" "
1929-30	P. Rous	D. Marine	" "	" "
1930-31	P. Rous	D. J. Edwards	" "	" "
1931-32	D. J. Edwards	A. R. Dochez	" "	" "
1932-34	A. R. Dochez	E. L. Opie	" "	" "
1934-36	E. L. Opie	P. E. Smith	" "	" "
1936-37	P. E. Smith	E. F. DuBois	" "	" "
1937-39	H. S. Gasser	J. T. Wearn	" "	" "
1939-40	J. T. Wearn	C. D. Leake	" "	" "
1940-41	J. T. Wearn	C. D. Leake	" "	" "
1941-42	W. deB. MacNider	C. H. Danforth	" "	" "

SECTIONAL MEETINGS AND MEMBERSHIP

Cleveland, Ohio

Chairman: J. A. Toomey. Secretary: H. D. Green. Members: 44.

Meetings: Western Reserve University, November 14, 1941

December 12, 1941

January 9, 1942

February 13, 1942

March 13, 1942

April 10, 1942

District of Columbia

Chairman: D. B. Jones. Secretary: H. M. Dyer. Members: 55.

Meetings: Georgetown University, December 4, 1941

February 15, 1942

George Washington University, April 29, 1942

Illinois

Chairman: G. E. Wakerlin. Secretary: L. V. Domm. Members: 141.

Meetings: University of Chicago, October 21, 1941

December 9, 1941

January 20, 1942

March 3, 1942

May 19, 1942

Iowa

Chairman: T. L. Jahn. Secretary: C. A. Winter. Members: 38.

Meetings: State University of Iowa, November 19, 1941

February 17, 1942

April 28, 1942

May 20, 1942

Minnesota

Chairman: F. H. Scott. Secretary: F. H. Scott. Members: 54.

Meetings: University of Minnesota, November 19, 1941

January 21, 1942

February 18, 1942

March 18, 1942

May 20, 1942

Missouri

Chairman: A. S. Gilson, Jr. Secretary: L. R. Jones. Members: 55.

Meetings: St. Louis University Medical School, December 10, 1941

Washington University Medical School, March 11, 1942

St. Louis University Medical School, May 13, 1942

New York

Chairman: M. Heidelberger. Secretary: J. A. Shannon. Members: 472.

Meetings: Cornell Medical College, November 12, 1941

New York University Medical College, February 25, 1942

New York Academy of Medicine, May 13, 1942

Pacific Coast

Chairman: C. A. Kofoed. Secretary: C. Weiss. Members: 104.

Meetings: University of California, October 4, 1941

Stanford University, December 3, 1941

University of California, February 4, 1942

Mount Zion Hospital, March 4, 1942

Stanford University, May 2, 1942

Peiping, China

Chairman: A. B. Fortuyn. Secretary: F. T. Chu. Members: 30.

Rocky Mountain

Chairman: H. S. Wilgus, Jr. Secretary: H. J. Clausen. Members: 20.

Meetings: University of Utah, November 1, 1941

University of Colorado, February 21, 1942

Colorado State College, May 13, 1942

Southern

Chairman: W. A. Sodeman. Secretary: K. L. Burdon. Members: 46.

Meetings: Tulane University, November 28, 1941

Louisiana State University, May 22, 1942

Southern California

Chairman: M. S. Dunn. Secretary: C. A. G. Wiersma. Members: 46.
 Meetings: University of Southern California, October 23, 1941
 California Institute of Technology, January 14, 1942
 Los Angeles Co. Med. Ass'n, March 17, 1942
 University of California, Los Angeles, May 21, 1942

Western New York

Chairman: E. F. Adolph. Secretary: H. C. Hodge. Members: 64.
 Meetings: Clifton Springs Sanitarium, October 18, 1941
 University of Buffalo, December 13, 1941
 University of Rochester, February 14, 1942

Wisconsin

Chairman: R. M. Meyer. Secretary: H. P. Rusch. Members: 43.
 Meetings: University of Wisconsin, December 9, 1941
 May 11, 1942

MEMBERSHIP

Members, March 31, 1941.....	1549	
Elected during year.....	106	
Total		1655
Resignations	7	
Arrears	6	
Deaths	17	
		30
Total Membership, March 31, 1942.....		1625
Membership: 1932 1942		
1138 1625		
Subscriptions, March 31, 1942.....		620

DEATHS OF MEMBERS

The Council records with regret the deaths of the following members:
 W. J. Atwell, K. Blackfan, M. Bodansky, A. J. Clark, C. L. Connor,
 C. W. Edmunds, J. E. Guberlet, L. Kast, J. E. Kemp, M. Kriss, F. B.
 Mallory, E. Megrail, E. B. Meigs, E. S. Miller, E. Pribram, C. J. Stucky,
 S. Weiss.

TREASURER'S REPORT

April 1, 1941-April 1, 1942

Balance on hand, April 1, 1941.....	\$ 6,320.99
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Income

Dues	\$ 7,065.29	
Reprints	3,643.36	
Space	2,723.68	
Cuts	705.74	
Changes	65.53	
Subscriptions	5,289.41	
Back No.	1,008.25	
Interest from special account.....	2.26	
		<hr/> \$20,503.52
Endowment Fund Temporarily in Corn Exchange Bank		749.29
		<hr/>
Total Cash Available, April 1, 1941-42.....		\$27,573.80

Disbursements

Printing	\$ 9,486.25	
Reprints	3,449.61	
Cuts	789.38	
		<hr/> \$13,725.24
Office Supplies, Telephone and Postage.....	1,166.97	
Salary	2,205.00	
Storage and Insurance	43.00	
Miscellaneous	195.44	
		<hr/> \$ 3,610.41
		<hr/> \$17,335.65
Transfer of Endowment Fund from Corn Exchange Bank		515.20
		<hr/>
		\$17,850.85
Cash Balance, April 1, 1942		9,722.95
		<hr/>
		\$27,573.80

Income (net)	\$20,503.52
Disbursements (net)	17,335.65
	<hr/>
Surplus	\$ 3,167.87
Accounts receivable—\$1,056.91	
Accounts payable None	

FUNDS

Endowment Fund

April 1, 1941	\$18,413.52	
Interest to April 1, 1942	722.05	
		19,135.57

Invested in

New York Title and Mortgage Co.....	\$ 5,640.00	
Title Guarantee and Trust Co.....	2,000.00	
Lawyers Mortgage Co.	1,500.00	
Bowery Savings Bank.....	1,549.79	
United States Savings Bonds	3,975.00	
Industrial Bonds	3,721.49	
Temporarily in Corn Exchange Bank.....	749.29	
		\$19,135.57

Surplus Fund

April 1, 1941	\$11,389.09	
Interest to April 1, 1942.....	392.40	
		\$11,781.49

Invested in

Title Guarantee and Trust Co.....	\$ 2,793.60	
Harlem Savings Bank	1,163.70	
United States Savings Bonds	1,950.00	
Industrial Bonds	5,874.19	
		\$11,781.49

Life Membership Fund

Invested in R. R. Federal Savings and Loan.....	\$ 75.00
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Auditors' Report

We have examined the books of the Society and believe the records of financial transactions to be accurately set forth in the Treasurer's report. We feel that the Society is to be congratulated not only on its financial condition but on its good fortune in benefiting by the self-sacrificing services of Doctor Goldforb and Miss McCabe.

(Signed) HANS T. CLARKE
VINCENT DU VIGNEAUD
ROBERT F. LOEB

June 1, 1942

MEMBERS' LIST

HONORARY MEMBERS

Flexner, Simon.....	Rockefeller Inst.
Howell, William H.....	Johns Hopkins Univ.
Porter, William.....	Harvard Univ.
Richet, Charles.....	Paris, France
Von Muller, Friedrich.....	Munich, Germany

MEMBERS

A bramson, D. I.....	May Inst. Med. Research, Cincinnati
Abramson, H. A.....	Coll. Physicians and Surgeons, New York
Abt, Arthur F.....	Northwestern Univ.
Adams, A. Elizabeth.....	Mount Holyoke Coll.
Addis, Thomas.....	Stanford Univ. Med.
Adlersberg, D.....	Beth Israel Hosp., N. Y.
Adolph, E. F.....	Univ. of Rochester Med.
Adolph, W. H.....	Ithaca, N. Y.
Alexander, Harry L.....	Washington Univ.
Allen, Bennet M.....	Univ. of Calif., L. A.
Allen, Edgar.....	Yale Univ.
Allen, William F.....	Univ. of Oregon
Alles, G. A.....	Pasadena, Calif.
Almquist, H. J.....	Univ. of Calif.
Alt, Howard L.....	Northwestern Univ. Med.
Althausen, T. L.....	Univ. of Calif. Med.
Altschule, M. D.....	Beth Israel Hosp., Boston
Altshuler, S. S.....	Wayne Univ. Med.
Alvarez, Walter C.....	Mayo Clinic
Alving, A. S.....	Univ. of Chicago
Amberg, Samuel.....	Mayo Clinic
Amberson, W. R.....	Univ. of Maryland Med.
Amoss, Harold L.....	Rockefeller Inst.
Anderson, Dorothy H.....	Coll. of Phys. and Surg., N. Y.
Anderson, H. H.....	Peiping Union Med. Coll.
Anderson, John E.....	Univ. of Minn.
Anderson, John F.....	E. R. Squibb & Son
Anderson, Rudolph J.....	Yale Univ.
Anderson, William E.....	Rockville, Conn.
Andrews, Edmund.....	Los Angeles, Calif.
Andrus, E. C.....	Johns Hopkins Univ.
Andrus, W. de W.....	Cornell Univ. Med. Coll.
Angevine, D. M.....	Wilmington, Del.
Ansbacher, Stefan.....	New York City
Antopol, William.....	Beth Israel Hosp., Newark, N. J.
Apperly, Frank L.....	Med. Coll. of Va.
Armstrong, Charles.....	National Inst. of Health, Washington
Armstrong, W. D.....	Univ. of Minn.

Arnold, Lloyd	Univ. of Ill.
Aronson, J. D.	Henry Phipps Inst.
Arnow, L. E.	Glenolden, Pa.
Asdell, S. A.	Cornell Univ.
Asher, Leon	Berne, Switzerland
Ashman, Richard	Louisiana State Univ.
Asmundson, V. S.	Univ. of Calif.
Atchley, D. W.	Presbyterian Hosp., N. Y. C.
Aub, Joseph C.	Mass. Gen. Hosp., Boston
Auer, John	St. Louis Univ.
Austin, J. Harold	Univ. of Pa.
Avery, B. F.	Boston Univ.
Avery, O. T.	Rockefeller Inst., N. Y. C.
Avery, Roy C.	Vanderbilt Univ.
Aycock, W. L.	Harvard Med.

B abkin, Boris P.	McGill Univ.
Bachem, Albert	Univ. of Ill. Med. Coll.
Baehr, George	Mt. Sinai Hosp., N. Y. C.
Bagg, Halsey J.	Memorial Hosp., N. Y. C.
Bahrs, Alice M.	Portland, Ore.
Bailey, Cameron V.	N. Y. Post-Graduate Med.
Bailey, P. V.	Univ. of Illinois Med.
Baitsell, George A.	Yale Univ.
Bakwin, Harry	N. Y. Univ. Med. Coll.
Baldwin, Francis M.	Univ. of S. Calif.
Ball, G. H.	Univ. of Calif., L. A.
Ball, H. A.	San Diego, Calif.
Balls, A. K.	U. S. Dept. of Agr.
Barach, Alvan L.	Coll. of Phys. and Surg., N. Y.
Barber, W. Howard	New York Univ. Med.
Barbour, Henry G.	Yale Univ.
Barer, Adelaide P.	State Univ. of Iowa
Barker, S. B.	Univ. of Tenn.
Barlow, O. W.	Rensselaer, N. Y.
Barnes, R. H.	Univ. of Minn.
Barnett, George D.	Stanford Univ.
Barr, David P.	Cornell Med. Coll.
Barron, E. S. G.	Univ. of Chicago
Barth, L. G.	Columbia Univ.
Bartley, S. H.	Washington Univ.
Bass, Charles	Tulane Univ.
Bast, T. H.	Univ. of Wisconsin
Bates, R. W.	Detroit, Mich.
Batterman, R. C.	New York Univ. Med.
Bauer, J. H.	Rockefeller Inst.
Bauman, Louis	Presbyterian Hosp., N. Y. C.
Baumann, E. J.	Montefiore Hosp., N. Y. C.
Baumberger, J. Percy	Stanford Univ.
Bayne-Jones, S.	Yale Univ.
Bazett, H. C.	Univ. of Pa.

Bean, John W.	Univ. of Mich.
Beard, H. H.	Louisiana State Univ.
Beard, J. W.	Duke Univ.
Beard, P. J.	Stanford Univ.
Beck, Claude S.	Western Reserve Univ.
Becker, E. R.	Iowa State Coll.
Beckman, Harry	Marquette Univ. Med.
Beckwith, T. D.	Univ. of Calif., L. A.
Behre, Jeannette A.	New York City
Belding, David L.	Boston Univ.
Bender, M. B.	Mt. Sinai Hosp., N. Y.
Bengston, Ida A.	National Inst. of Health, Washington
Berg, B. N.	Columbia Univ.
Berg, C. P.	State Univ. of Iowa
Berg, William N.	N. Y. City
Bergeim, Olaf	Univ. of Ill.
Bergmann, Max	Rockefeller Inst.
Bernhard, Adolph	Lenox Hill Hosp., N. Y. City
Bernhart, F. W.	Cleveland, O.
Bernthal, T. G.	Vanderbilt Univ.
Berry, George P.	Univ. of Rochester Med.
Beutner, R.	Hahnemann Med. Coll., Philadelphia
Bierman, W.	Mt. Sinai Hosp., N. Y.
Bieter, Raymond N.	Univ. of Minn.
Bills, C. E.	Mead, Johnson and Co., Evansville, Ind.
Bing, Franklin C.	Am. Med. Assn., Chicago
Bing, R. J.	N. Y. Univ. Med.
Birkhaug, Konrad E.	Geofysisk Inst., Bergen, Norway
Birnbaum, G. L.	New York Med. Coll.
Bishop, George H.	Webster Groves, Mo.
Biskind, G. R.	Mt. Zion Hosp., San Francisco
Blair, John E.	Hosp. for Joint Diseases, N. Y.
Blake, F. G.	Yale Univ.
Blalock, Alfred	Vanderbilt Univ. Med.
Blatherwick, Norman R.	Metropolitan Life Insurance Co., N. Y. City
Blinks, L. R.	Stanford Univ.
Bloch, Robert G.	Univ. of Chicago
Block, Richard J.	N. Y. State Psychiatric Inst.
Bloom, William	Univ. of Chicago
Bloomfield, A. L.	Stanford Univ. Med.
Bloor, W. R.	Univ. of Rochester
Blount, R. F.	Univ. of Minn.
Blum, Harold F.	National Cancer Inst.
Blumberg, Harold	Johns Hopkins Univ.
Blumgart, H. L.	Beth Israel Hosp., Boston
Bock, Joseph C.	Marquette Univ.
Bodansky, A.	Hosp. for Joint Diseases, N. Y.
Bodansky, Oscar	New York Univ.
Bodine, J. H.	State Univ. of Iowa
Bogen, Emil	Olive View, Calif.
Boissevain, Charles H.	Colorado Coll.

Bollman, Jesse L.	Mayo Clinic
Bonner, James	Calif. Inst. of Technology
Booher, Lela E.	Milwaukee, Wisc.
Boor, Alden K.	Univ. of Chicago
Boothby, Walter M.	Kahler Hosp., Rochester, Minn.
Boots, Ralph H.	Presbyterian Hosp., N. Y.
Borsook, Henry	Calif. Inst. of Technology
Bowen, B. D.	Buffalo Gen. Hosp.
Boyce, F. F.	New Orleans, La.
Boyd, Eldon M.	Queens Univ., Canada
Boyd, Theo. E.	Loyola Univ.
Boyd, E. A.	Univ. of Minn. Med.
Bozler, Emil	Ohio State Univ.
Bradford, William L.	Univ. of Rochester
Bradley, H. C.	Univ. of Wisconsin
Brand, Erwin	Coll. of Phys. and Surg., N. Y.
Branham, Sara E.	National Inst. of Health, Washington
Brewer, George	Univ. of Pa.
Brewer, Robert K.	Syracuse Univ.
Briggs, A. P.	Univ. of Georgia
Brinkhous, K. M.	Danville, Ky.
Bronfenbrenner, J.	Washington Univ.
Bronk, D. W.	Univ. of Penn.
Brooks, Clyde	Louisiana State Univ.
Brooks, Matilda M.	Univ. of Calif.
Brooks, S. C.	Univ. of Calif.
Broun, G. O.	St. Louis Univ. Med.
Brown, J. Howard	Johns Hopkins Univ.
Brown, John B.	Ohio State Univ.
Brown, Rachel	N. Y. State Dept. of Health
Brown, Wade H.	Rockefeller Inst., Princeton
Browne, J. S. L.	Royal Victoria Hosp., Montreal
Bruger, Maurice	N. Y. Post-Graduate Med.
Brunschwig, Alexander	Univ. of Chicago
Buchanan, A. R.	Univ. of Colo.
Buchanan, Robert E.	Iowa State Coll.
Buehbinder, W. C.	Michael Reese Hosp., Chicago
Bueding, Ernest	N. Y. Univ. Med.
Buell, Mary V.	Johns Hopkins Univ.
Bulger, H. A.	Washington Univ.
Bullowa, J. G. M.	Harlem Hosp., N. Y.
Bunney, W. E.	E. R. Squibb & Sons
Bunting, C. H.	Univ. of Wisc.
Burch, George E.	New Orleans, La.
Burch, John C.	Vanderbilt Univ. Med.
Burdon, Kenneth L.	Louisiana State Univ.
Burk, Dean	National Cancer Inst.
Burky, Earl L.	Johns Hopkins Hosp.
Burns, E. L.	Louisiana State Univ.
Burns, Robert K., Jr.	Carnegie Inst., Baltimore, Md.
Burr, George O.	Univ. of Minn.

Burrows, M. T.	Pasadena, Calif.
Burrows, William	Univ. of Chicago
Burstein, C. L.	N. Y. Univ. Med.
Butcher, E. O.	Hamilton Coll.
Butt, E. M.	Univ. of So. Calif.
Butts, Joseph S.	Oregon State Coll.
Byerly, T. C.	U. S. Animal Exp. Farm, Beltsville, Md.
Byrne, Joseph	Fordham Univ.

C ahill, W. M.	Wayne Univ.
Calkins, Gary N.	Columbia Univ.
Cameron, A. T.	Univ. of Manitoba
Cannan, Robert K.	N. Y. Univ.
Cannon, Paul R.	Univ. of Chicago
Cannon, Walter B.	Harvard Med.
Cantarow, Abraham	Jefferson Med. Coll.
Carey, E. J.	Marquette Univ.
Carlson, A. J.	Univ. of Chicago
Carmichael, E. B.	Univ. of Alabama Med.
Carmichael, L.	Tufts Coll.
Carpenter, C. M.	Univ. of Rochester
Carr, C. J.	Univ. of Md.
Carr, J. L.	Univ. of Calif. Hosp.
Carruthers, A.	Birmingham, England
Cary, C. A.	U. S. Dept. of Agriculture
Casey, Albert E.	Louisiana State Univ.
Cash, James R.	Univ. of Va.
Casida, L. E.	Univ. of Wisc.
Castaneda, M. R.	Hosp. General, Mexico City
Cattell, McKeen	Cornell Univ. Med. Coll.
Cecil, R. L.	Cornell Univ. Med. Coll.
Cerecedo, L. R.	Fordham Univ.
Chadwick, C. S.	Vanderbilt Univ.
Chaikoff, I. L.	Univ. of Calif.
Chambers, Robert	New York Univ.
Chambers, William H.	Cornell Univ. Med. Coll.
Chang, Hsi Chun	Peiping Union Med. Coll.
Chang, Hsiao-Chien	Hunan, China
Chargaff, Erwin	Coll. of Phys. and Surg.
Charipper, H. A.	New York Univ.
Cheer, S. N.	W. China Union Univ.
Chen, Graham M.	Chicago, Ill.
Chen, K. K.	Eli Lilly and Co., Indianapolis
Chen, T. T.	Peiping Union Med. Coll.
Cheney, R. H.	Long Island Univ.
Chidester, F. E.	Toronto, Can.
Child, C. M.	Stanford Univ.
Chittenden, R. H.	Yale Univ.
Chouke, K. S.	Univ. of Pa.
Chow, B. F.	Squibb Inst., New Brunswick, N. J.

Christensen, K.	St. Louis Univ.
Christian, Henry A.	Peter Bent Brigham Hosp.
Christman, Adam A.	Univ. of Mich.
Chu, F. T.	Peiping Union Med. Coll.
Chung, H. L.	Peiping Union Med. Coll.
Clark, Ada R.	Coll. of Phys. and Surg.
Clark, George	Med. Coll. of S. Carolina
Clark, Guy W.	Lederle Lab., Pearl River, N. Y.
Clark, P. F.	Univ. of Wise.
Clark, W. G.	Univ. of Minn.
Clarke, Hans T.	Coll. of Phys. and Surg.
Claude, A.	Rockefeller Inst.
Clausen, H. J.	Univ. of Colo. Med.
Claussen, S. W.	Strong Memorial Hosp., Rochester, N. Y.
Clawson, Benjamin J.	Univ. of Minn.
Clifton, Charles E.	Stanford Univ.
Climenko, D. R.	Rensselaer, N. Y.
Clowes, G. H. A.	Eli Lilly and Co., Indianapolis
Coca, A. F.	Oradell, N. J.
Code, C. F.	Mayo Foundation
Coggeshall, L. T.	Univ. of Mich.
Cohen, Barnett	Johns Hopkins Med.
Cohen, Milton B.	St. Alexis Hosp., Cleveland
Cohn, A. E.	Rockefeller Inst., N. Y.
Cohn, Isidore	New Orleans, La.
Cole, Arthur G.	Univ. of Ill. Med.
Cole, Harold H.	Univ. of Calif., Davis
Cole, L. J.	Univ. of Wise.
Cole, Rufus I.	Rockefeller Inst., N. Y. City
Cole, Warren H.	Univ. of Ill. Med.
Cole, William H.	Rutgers Univ.
Collens, William S.	Brooklyn, N. Y.
Collier, William D.	St. Elizabeth's Hosp., Youngstown, O.
Collins, D. A.	Temple Univ.
Collip, J. B.	McGill Univ.
Compere, E. L.	Univ. of Chicago
Conklin, E. G.	Princeton Univ.
Cook, Charles A.	Burroughs Wellcome and Co.
Cook, Donald H.	School of Tropical Med., San Juan, P. R.
Cooke, J. V.	Washington Univ.
Coombs, Helen C.	Brooklyn Coll.
Cooper, Frank B.	W. Penn. Hosp., Pittsburgh
Cooper, Merlin L.	Univ. of Cincinnati
Cope, O. M.	N. Y. Med. Coll.
Copenhaver, W. M.	Columbia Univ.
Corbin, Kendall B.	Univ. of Tenn.
Corey, E. L.	Univ. of Va.
Cori, Carl F.	Washington Univ.
Corley, Ralph C.	Purdue Univ.
Corner, George W.	Carnegie Inst., Baltimore
Corper, H. J.	National Jewish Hosp., Denver, Colo.

Co Tui.....	N. Y. Univ. Med. Coll.
Coulson, E. J.....	U. S. Dept. of Agriculture
Cowdry, E. V.....	Washington Univ.
Cowgill, George R.....	Yale Univ.
Cox, Herald R.....	U. S. Public Health Inst., Hamilton, Mont.
Cox, Warren M., Jr.....	Mead Johnson Co.
Cram, Eloise B.....	Nat. Inst. of Health, Washington
Crampton, C. Ward.....	N. Y. Post-Graduate Med.
Crandall, L. A., Jr.....	Univ. of Tenn.
Creaser, C. W.....	Wayne Univ. Med.
Crile, George W.....	Western Reserve Univ.
Crittenden, Phoebe J.....	George Washington Univ.
Crohn, Burrill B.....	Mt. Sinai Hosp., N. Y.
Csonka, F. A.....	U. S. Dept. of Agr., Washington, D. C.
Culler, E. A.....	Univ. of Rochester
Cummins, Harold.....	Tulane Univ.
Cunningham, Bert.....	Duke Univ.
Curtis, G. M.....	Ohio State Univ.
Curtis, Maynie R.....	Columbia Univ.
Cutler, Elliott C.....	Peter Bent Brigham Hosp., Boston
Cutting, W. C.....	Stanford Univ. Med.
Cutuly, Eugene.....	Wayne Univ. Med.

D ack, Gail M.....	Univ. of Chicago
Dakin, H. D.....	Ossining, N. Y.
Dalldorf, Gilbert.....	Grasslands Hosp., Valhalla, N. Y.
Dalton, A. J.....	McGill Univ.
D'Amour, F. E.....	Univ. of Denver
Danforth, Charles H.....	Stanford Univ.
Danforth, D. N.....	Sloane Hosp., N. Y. City
Daniel, J. Frank.....	Univ. of Calif.
Daniels, Amy L.....	Avon, Conn.
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